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DESIGN AND SYNTHESIS OF ANTITHROMBOTIC LIPOSOMAL PROTEIN CONJUGATE

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DESIGN AND SYNTHESIS OF ANTITHROMBOTIC LIPOSOMAL PROTEIN CONJUGATE

HAILONG ZHANG

ABSTRACT

Recent advances in the molecular bases of haemostasis have highlighted that endothelial thrombomodulin (TM) plays a critical role in local haemostasis by binding thrombin and subsequently converting protein C to its active form (APC). In addition, the binding of thrombin to TM drastically alters the thrombin's procoagulant activities to anticoagulant activities. The lipid bilayer in which it resides serves as an essential 'cofactor', locally concentrating and coordinating the appropriate alignment of reacting cofactors and substrates for protein C activation. On the other hand, liposomes have been extensively studied as cell membrane model as well as carrier for delivering certain vaccines, enzymes, drugs, or genes. In this study, antithrombotic liposomal TM conjugate was design and developed by combination of antithrombotic membrane protein TM into liposome through recombinant and bioorthogonal conjugation techniques, which providing a rational strategy for generating novel and potential antithrombotic agent. Namely, the liposomal TM conjugate mimics the native endothelial antithrombotic

mechanism of both TM and lipid components and thus is more forceful than current antithrombotic agent.

First, an efficient and chemoselective liposome surface functionalization method was developed based on Staudinger ligation, in which a model compound carbohydrate derivative carrying a spacer with azide was conjugated onto the surface of preformed liposomes carrying a terminal triphenylphosphine in PBS buffer (pH 7.4) and at room temperature.

Second, recombinant TM containing the EGF-like domains 456 with an azidohomoalaine at the C-terminal has been expressed and incorporated into liposome to form antithrombotic liposomal TM conjugate via Staudinger ligation. In addition, another chemically selective liposomal surface functionalization method for antithrombotic liposomal TM conjugated has been developed based on copper-free click chemistry, which provides an alternative approach to improving reaction efficiency and stability and represents an optimal platform for exploring membrane protein's activity of TM.

In addition, chemically selective liposomal surface functionalization and liposomal microarray fabrication using azide-reactive liposomes has been developed and confirmed by fluorescence imaging, fluorescent dye releasing kinetics, and AFM techniques. The azide-reactive liposome provides a facile strategy for membrane-mimetic glyco-array fabrication, which may find important biological and biomedical applications such as studying carbohydrate–protein interactions and toxin and antibody screening.

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NOMENCLATURE

TM	Thrombomodulin
APC	Activated Protein C
PAR	Protease Activated Receptor
EPCR	Endothelial Cell Protein C Receptor
DPPC	Dipalmitoyl Phosphatidil Choline
DSPC	1, 2-disteroyl-sn-glycero-3-phosphocholine
DSPE	1, 2-disteroyl-sn-glycero-3-phosphoethanolamine
DPPE	1, 2-dipalmitoyl- <i>sn</i> -glycero-3-phosphoethanolamine
TP	Triphenylphosphine
PEG	Polyethylene Glycol
UAA	Unnatural Amino Acid
DCC	Dicyclohexylcarbodiimide
NHS	N-hydroxysuccinimide
DLS	Dynamic Light Scattering
DBCO	Dibenzylcyclooctynephenyl

CHAPTER I

INTRODUCTION

1.1. Thrombosis and endothelial thrombomodulin (TM)

Thromboembolic diseases such as myocardial infarction, stroke, and thromboembolism are major public health problems with significant mortality and morbidity in the United States [1]. It has been considered that all these diseases are primarily disorder of platelet function and the coagulation. In the past decades, antiplatelet agents such as aspirin and ticlopidine and anticoagulant agents such as heparin and warfarin have been established and provided remarkable achievements in the treatment of thromboembolic diseases. However, these drugs have considerable limitations. In particular, the complications associated with bleeding represent a major drawback. Heparin and warfarin require laboratory monitoring, which are inconvenient to both patients and physicians and are costly. Furthermore, no beneficial effects in preventing restenosis after revascularization procedures have yet been obtained with the established antithrombotic agents.

Recent understanding of haemostasis in the molecular bases has highlighted new targets for novel antithrombotic agent design. For example, antithrombotics aiming at

inactivating thrombin, inhibiting thrombin generation, inhibiting fibrin formation, inactivating coagulation cascade factors, destroying fibrin and interrupting platelet activity have been investigated widely [2]. In particular, selective inhibitors of thrombin and factor Xa, and components of the anticoagulant protein C system (recombinant activated human protein C or human soluble thrombomodulin) have been extensively studied. Some of these agents have had promising results in Phase III studies but still with limitations above. Given the limitations of the current antithrombotic agents, development of new antithrombotics with improved antithrombotic activity and reduction of unwanted bleeding and easier patient management is highly demanded.

1.1.1 Endothelial TM Plays a Central Role in Local Haemostasis

Thrombosis results from a failure of haemostatic balance, which is normally maintained by a complex series of coagulation reactions that involve both systemic and local factors [3]. The endothelium contributes to local haemostatic balance by producing thrombomodulin (TM), which functions as an essential cofactor for the activation of anticoagulant protein [4]. TM modulates the activity of thrombin from a procoagulant to an anticoagulant protease [5]. To date, TM not only is a natural anticoagulant but also functions in anti-inflammatory (Figure 1.1) [6]. The anticoagulant function of TM was performed through protein C anticoagulant systems. In the protein C pathway, protein C will be activated to form activated protein C (APC) by thrombin, in which the speed of activation is very slow. After binding with TM on the surface of endothelial membrane, thrombin immediately enhances protein C activation by 1000 fold. On the one hand, APC produced in the pathway performed anticoagulant by inhibition of thrombin. Briefly,

APC binds protein S to form new complex after dissociating from endothelial cell protein C receptor (EPCR), which prevents thrombin generation by destroying factor FVa and FVIIIa. At same time, the inhibition of thrombin induces an indirect inflammation by preventing activation of protease-activated receptor (PAR)-1. On the other hand, APC-EPCR complex anti-inflammatory effects via direct modulation of PAR-1[7].

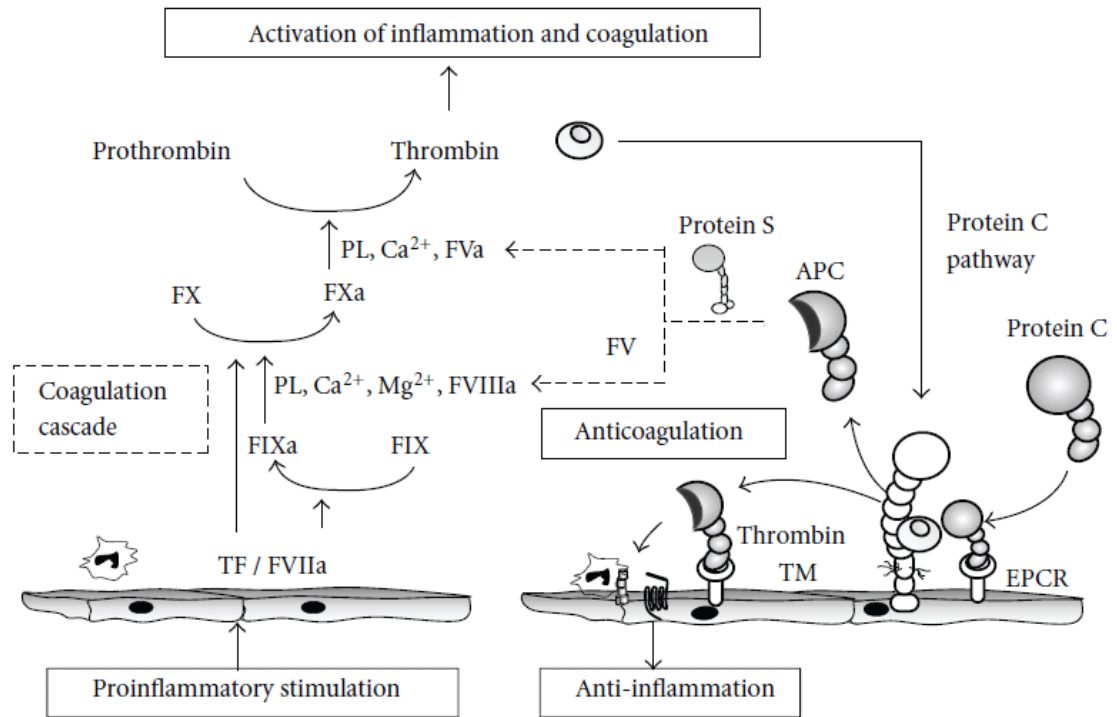


Figure 1.1 Crosstalk Between Inflammation and Coagulation. Thrombin acts to form a positive feedback loop that augments coagulation and inflammation. In a negative feedback loop, the thrombin-TM complex activates protein C and downregulates coagulation and inflammation. APC, in tandem with its cofactor protein S, inactivates FVa and FVIIIa. In addition, the APC-EPCR complex induces anti-inflammatory effects through PARs. Therefore, thrombin, TM, and protein C-EPCR play pivotal roles in the crosstalk that occurs between inflammation and coagulation. Reproduced from [6]

1.1.2 TM Structure and Function

As a natural anticoagulant protein, human TM, consisting of 557 amino acids, is mostly expressed on the surface of endothelium cell membranes and is structurally divided into five consecutive domain from amino terminal (Figure 1.2) [5]. C-type lectin domain, from N-terminal of TM, is responsible for mediating anti-inflammatory activities [8]. Adjacent to the lectin like domain is the epidermal growth factor (EGF)-like domain consisting of six repeat, which plays a key role in protein C activation and anticoagulant activity of TM. Although there remains unknown function for the first two EGF-like repeats (1-2), another three EGF-like repeats (4-6) have been studied very extensively and are responsible for whole activation of protein C [9]. Actually, EGF-like repeats 5-6 do not involve protein C activation by itself, but are necessary for binding thrombin to TM via thrombin's anion-binding exosite I. Differently, EGF like repeats 3-6 are responsible for activation of thrombin activatable fibrinolysis inhibitor (TAFI) [10]. After EGF like domain, there is O-linked glycosylation domain composing of serine/threonine, in which chondroitin sulfate is attached to TM to enhance protein C activation of TM by strengthening the thrombin-TM binding at anion-binding exosite 2 of Thrombin [11]. Next, a membrane spanning region consisting of 23 hydrophobic amino acids is linked to o-glycosylation domain. Finally, a short cytoplasmic domain containing a free cysteine is attached to C terminal in which cysteine may be responsible for meditation of multimerization to TM [12].

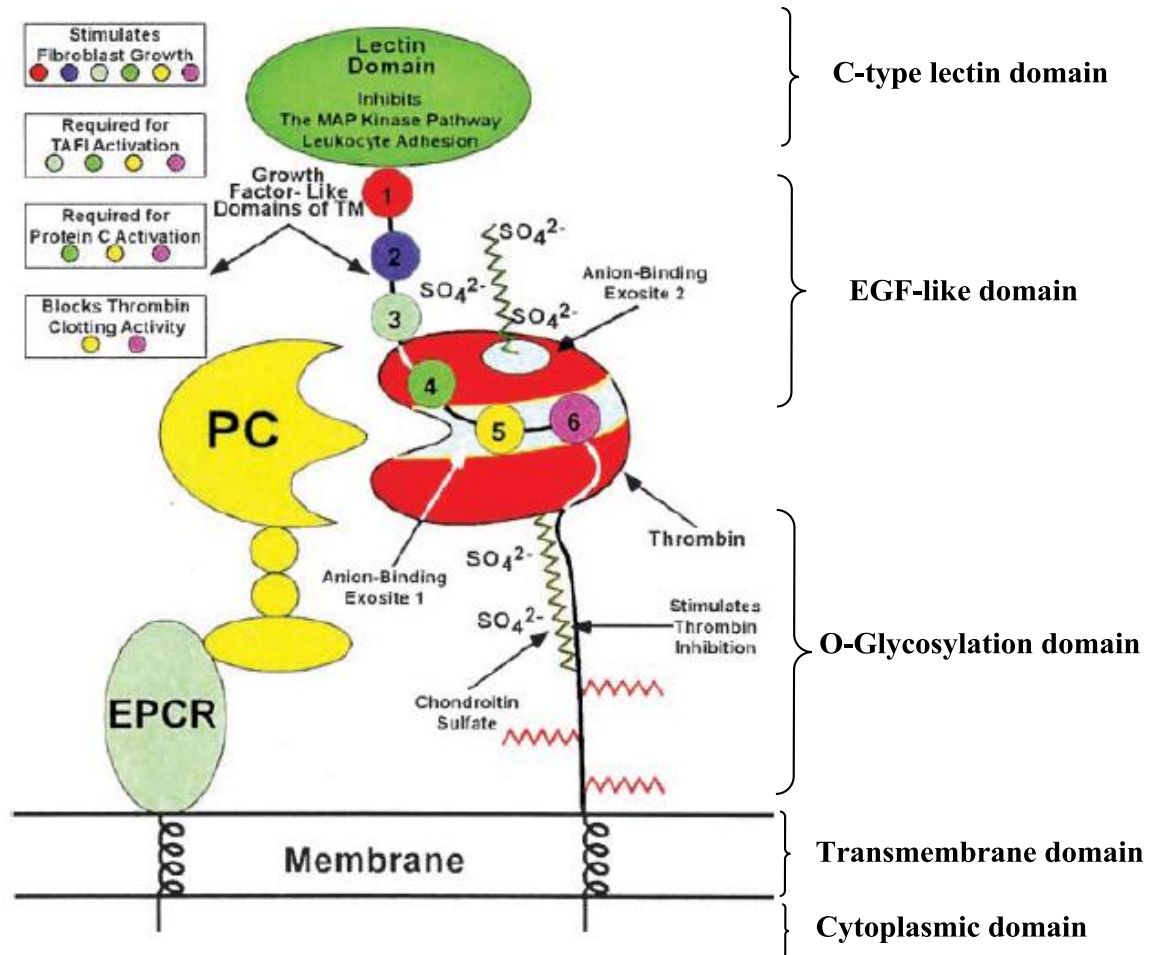


Figure 1.2 TM Structure and the Model of Protein C Activation Complex. Thrombin binding to thrombomodulin involves anionbinding exosite 1 on thrombin and EGF domains 4 to 6 on thrombomodulin. A chondroitin sulfate moiety on thrombomodulin increases the affinity for thrombin by binding anion-binding exosite 2 on thrombin. The activation of protein C to APC by the thrombin-thrombomodulin complex is enhanced by binding to EPCR. Reproduced from [13].

1.1.3 Current Research on Exploring on the Therapeutic Applications of Recombinant TM

It has been known that thromboembolic disorders have been found to result from relative deficiency of TM [14] or protein C [15], and homozygous deficiency of TM has

even been shown to confer lethality in mice [16]. The conversion of protein C to APC may be impaired during sepsis because of the down-regulation of TM by inflammatory cytokines [17]. The decrease in TM expression is associated with a local inflammatory response [18], and transcription of the TM gene is known to be negatively regulated by inflammatory cytokines such as tumor necrosis factor (TNF)- α or interleukin-1 β [19]. An alternative possibility is that TM may be shed from the endothelial surface by proteases produced by activated leukocytes [20]. It has been demonstrated that local over-expression of TM through adenoviral gene transfer can prevent thrombosis in an animal model [21]. However, gene therapies currently are not suitable to manage acute cardiovascular conditions.

Recent studies have shown that recombinant human soluble TM (rhsTM, the extra cellular portion of TM) has a long biological half-life in the absence of significant systemic coagulation and produces dose-dependent anticoagulant and antithrombotic effects in both rodents and primates [22-25]. In tissue factor-infused monkeys, rhsTM pretreatment decreases the concentration of thrombin–antithrombin complexes in a dose-dependent manner. Recombinant soluble TM is anticoagulant to various degrees in normal and protein C-depleted plasma [26]. A most recent study with a primate model demonstrated the antithrombotic effects of rhsTM were largely intact in the presence of protein C blocking antibody [27]. This study showed that pharmacological doses of rhsTM directly inhibited the prothrombotic response (thrombin generation) and upregulation of antithrombotic system (APC generation). This is in contrast to the conclusions of previous studies, which attributed the antithrombotic effects of rhsTM primarily to APC generation [28]. Therefore, rhsTM might exert anticoagulant effects by

at least two mechanisms: (1) directly, *via* direct thrombin inhibition and (2) indirectly, *via* supporting the thrombin/thrombomodulin-catalyzed local activation of protein C at the site of thrombin generation.

1.1.4 Lipid Membranes Play a Pivotal Role in Regulating Blood Coagulation Reactions

Both procoagulant and anticoagulant reactions occur at a physiologically significant rate only when the respective enzymes form multicomponent complexes on lipid membrane surfaces [29]. It has been observed that lipid vesicles containing negatively charged phospholipids, especially phosphatidylserine (PS), markedly enhance the rate of activation of procoagulant and anticoagulant enzymes [30, 31]. TM is a membrane protein of endothelium. The lipid bilayer in which it resides serves as an essential ‘cofactor’, locally concentrating and coordinating the appropriate alignment of reacting cofactors and substrates for protein C activation (Figure 1B). In concert with TM, the lipid membrane accelerates protein C activation and subsequently optimizes APC anticoagulant activity. Esmon et al. had demonstrated that TM incorporated into lipid vesicles resulted in substantially enhanced protein C activation [32]. The K_m for protein C obtained with TM incorporated into lipid vesicles is very similar to that obtained on the endothelial cell surface. Studies also suggested that protein C prefers binding onto lipid membrane containing both phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [33, 34]. On the other hand, efficient inactivation of Factor Va by APC requires the presence of negatively charged phospholipids, with phosphatidylserine (PS) being the most potent stimulator of the APC cleavage of Factor

Va [31]. In addition, incorporation of phosphatidylethanolamine (PE) into the phospholipid membrane has been found to enhance the inactivation of Factor Va by APC [35]. Therefore, lipid membrane is necessary to preserve TM's activity and the lipid membrane recognition is necessary for both protein C activation and Factor Va inactivation and shares many properties in common with the endothelial cell surface.

1. 2 Liposomes as Drug Delivery Vehicles

1.2.1 Liposomes

Liposomes, artificial microscopic and self-closed vesicles consisting of bilayer phospholipids membrane as model of biological cell membrane, have been studied extensively during the past few decades as drug delivery systems with great potential. Lipids as parts of natural membranes in live organism exhibit low immunogenicity and toxicity, good biocompatibility and biodegradability [36] and has been considered as a useful tool in genetic engineering and diagnostic techniques [37]. Additionally, due to their structure, chemical composition and colloidal size, liposomes are easily prepared as controlled size from microscale to nanoscale and surface functionalization through chemical biology with carbohydrate, peptide and protein [38-40].

Normally, liposomes are classified according to size and lamellarity. Small unilamellar vesicles (SUV) consist of a single lipid bilayer with 25-100 nm in size; large unilamellar vesicles (LUV) shows a diameter of 100-400 nm; multilamellar vesicles (MLV) consist of two or more concentric bilayers with a size range of 200 nm to several microns; multivesicular vesicles (MVV) usually have multicompartmental structure with more than 1 micrometer in size [41]. As cell-like compartments, liposomes' size and

lamellarity with lipid composition play a vital role in modeling cell properties like the fluidity, permeability, stability [42]. In turn, liposomes could meet specific needs since the size, lamellarity and composition can be controlled and prepared by requirement.

Since liposome has been recognized 40 years ago, several generation of liposome based drug delivery systems have been developed to overcome drawbacks such as short life time or non-targeting and diverse pharmaceutical criteria (Figure 1.3) [43].

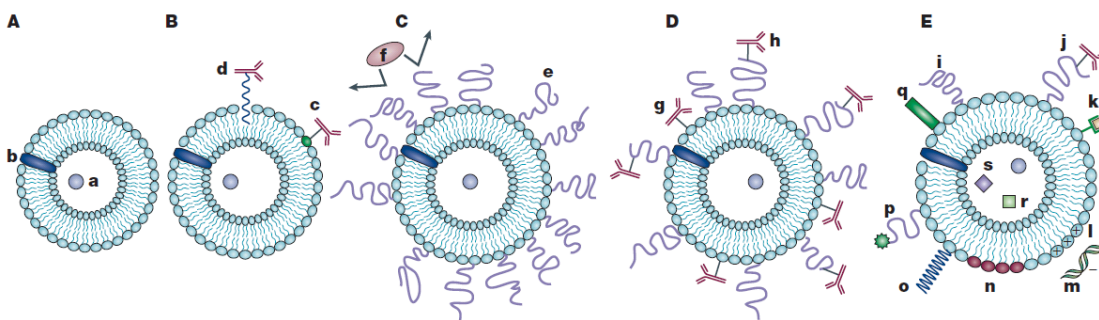


Figure 1.3 Evolution of Liposomes. A. Early traditional phospholipids ‘plain’ liposomes with water soluble drug in interior (a) or membrane (b). B. Antibody-targeted immunoliposome with antibody covalently coupled (c) or hydrophobically anchored (d). C. Long-circulating liposome grafted with a protective polymer (e) from opsonizing proteins (f). D. Long-circulating immunoliposome simultaneously bearing both protective polymer and antibody in the surface (g) or the chain end (h). E. New-generation liposome, the surface of which can be modified (separately or simultaneously) by different ways: protective polymer (i&o), antibody (j), diagnostic label (k), charged lipids (l&n), DNA (m), peptide (p), viral components (q), magnetic particles (r), gold or silver particles (s). traditional liposomes. Reproduced from [43].

Early traditional liposomes are mainly comprised of natural phospholipids or lipids such as dipalmitoyl phosphatidil choline (DPPC, PC or egg phosphcholine), monosialoganglioside. As the first generation of liposome, although early traditional

liposomes have attracted a lot of attention in basic science research [44], they are limited to further application in pharmaceutical industry and clinical setting due to poor stability, short half-time and toxicity in some systems [45-47]. Many attempts have been made to remove those drawbacks. For example, cholesterol was added to bilayer mixture to stabilize the structure of bilayer membrane [48]; liposome composed of egg lecithin was prepared by insertion of negative or positive charges to improve drug entrapment efficiency [49]; liposomes with different size range have been made to explore the effect of morphology of liposome on half-time of liposome in the systemic circulation [50]. However, the major problems such as fast elimination and non-targeting still remain unresolved.

1.2.2 Stealth Liposomes

First breakthrough in the liposome development is stealth liposome or long-circulating liposome. Stealth liposome strategy was achieved simply by coating the surface of liposome with a hydrophilic and biocompatible polymer-poly ethylene glycol (PEG). PEG provides a steric barrier that prevents liposome aggregation and opsonizing proteins (Figure 1.3 C) [51]. Many other hydrophilic polymers such as chitosan [52], silk-fibroin [53], and polyvinyl alcohol (PVA) [54] have been tried to increase half-time of liposome and reduce toxicity. Although those hydrophilic polymers could meet biodegradability, nontoxicity and lower immunogenicity, PEG still keep the gold standard in protecting liposome and increasing circulation time. Vaccines or anticancer therapeutics to non-viral gene therapy vectors sequesters part of the solvent, in which they freely float into their interior. In the case of one bilayer encapsulating the aqueous

core one speaks either of small or large unilamellar vesicles while in the case of many concentric bilayers one defines large multilamellar vesicles. All of which can be well controlled by preparation methods, liposomes exhibit several properties which may be useful in various applications. The most important properties are colloidal size, i.e. rather uniform particle size distributions in the range from 20 nm to 10 μ m, and special membrane and surface characteristics. They include bilayer phase behavior, its mechanical properties and permeability, charge density, presence of surface bound or grafted polymers, or attachment of special ligands, respectively. Additionally, due to their amphiphilic character, liposomes are a powerful solubilizing system for a wide range of compounds. In addition to these physico-chemical properties, liposomes exhibit many special biological characteristics, including (specific) interactions with biological membranes and various cells [55].

1.2.3 Targeted Liposome

The further development of liposome involves increasing liposomal drug accumulation in the desired tissues and organs, in other words, specific targeting of liposome based systems should be addressed (Figure 1.3 B, D&E). Overcoming the biological barriers between administration and the site of action of drug or gene is becoming increasingly important for the development of better and safer medicines. Small organic molecule-based drugs often suffer from poor bioavailability problems, such as limited water solubility and/or poor membrane permeability. Peptide, protein, or DNA-based biomacromolecules are even more challenging to develop safe and efficient drug products due to the size and poor biological stability as well as immunogenicity

problems [57]. Targeted liposome increases noticeably the local drug concentration in the ideal tissue or cells without harmful side effect on healthy cells or/and systemic side effect [58-60]. Different types of targeting moieties such antibodies, peptide, glycoprotein, oligopeptide, polysaccharide, growth factors, folic acid, carbohydrate, receptors, and peptides have been extensively explored to conjugate liposome to overcome these limitations in the past decades [61-64].

1.3 Current Methods for Liposome Surface Modification

Liposomes as mimics of cell membrane have attracted much attention. Conjugation different ligands such as proteins, peptides and drugs to liposomes has been studied extensively in numerous applications. A number of conjugation methods have been developed for liposome surface functionalization and are described in the following.

1.3.1 Direct Liposome Formation with Ligands

To most of small ligands, surface modification of liposome through direct liposome formation is common method. This strategy involves the initial synthesis of the key lipid-ligand conjugate, followed by formulation of the liposome with all lipid components. In this direct liposome formation method, around 50% of total ligands inevitably are facing the interior of liposome and thus become unavailable for their intended interaction with their target molecules. This approach is well suited for ligands that are cheap and in large quantities, such as saccharides [65] and vitamins [66].

1.3.2 Post-Insertion Approach

Another method for liposome surface modification was developed to overcome problems in surface modification through direct liposome formation. In this approach, liposome was prepared firstly, and then functionalized ligands with lipid tail will incorporate into performed liposome. Normally, targeted ligands will be covalently attached with lipids and then dissolved into buffer to form micelles. Next, micelles containing ligands will mix with performed liposome to allow ligands incorporate into performed liposome by fusion. Lipid-ligand conjugates normally have limited solubility and stability in solvent, or are incompatible with various stages of manufacture. The advantages of this approach for liposome surface modification include reducing ligands amounts, noncovalent and reversible incorporation and make it suitable to antibody [67], protein [68] and peptides [69] incorporation into the liposome surface. On the other hand, this approach suffers from low incorporation efficiency and limited solubility and stability of ligands conjugates with lipid in solvent, as a result, limits its applications.

1.3.3 Post-Functionalization Approach

Chemical modification methods, namely post-functionalization, which in most cases involve the coupling of biomolecules to the surface of preformed vesicles that carry functionalized (phospho) lipid anchors, have been developed [70]. So far, variable coupling methods for post functionalization such as amide or thiol-maleimide coupling as well as by imine or hydrazone linkage have been achieved. Below each coupling reaction for post liposome surface functionalization are described in detail (Figure 1.4).

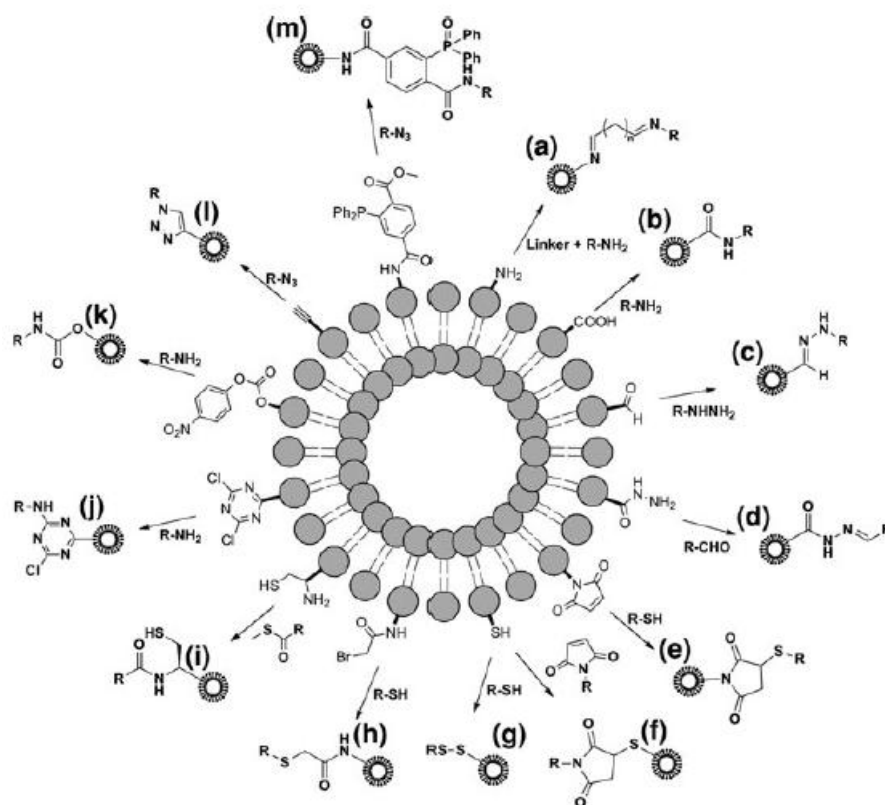


Figure 1.4 Schematic Illustration of the Various Coupling Methods that have been Developed in order to Post-Functionalized Liposomes: a Amine functionalization, b carboxylic acid functionalization, c aldehyde functionalization, d hydrazine functionalization, e maleimide functionalization, f thiol functionalization, g thiol functionalization (disulfide bond formation), h bromoacetyl functionalization, i cysteine functionalization, j cyanur functionalization, k p-nitrophenylcarbonyl functionalization, l alkyne functionalization, m triphenylosphine functionalization. Reproduced from [71].

1.3.3.1 Traditional Covalent Conjugation

Linkages containing amide bonds

The amide bond, generally represented as $RC(O)NH_2$, is very stable and widely used for surface modification by bioconjugation and becomes one of earliest methods to functionalize performed liposome. This linkage could be performed by coupling ligands

to amine functionalized liposomes or coupling ligands to carboxylic acid modified liposomes. Since liposome is kind of water solution, water soluble carbodiimides, such as 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride [EDC] was used to allow for an aqueous conjugation reaction. And N-hydroxysulfosuccinimide [Sulfo-NHS] is used to form a stable intermediate (NHS ester derivatives) and followed by formation of amide bonds with high efficiency.

Another popular approach through linkage containing amine bonds is to exploit the reaction between aldehyde and hydrazide group (Figure 1.4d). Normally, hydrazide will be incorporated into liposome to attain hydrazide functionalized liposome which will react with aldehyde group in antibody or protein. This coupling is specially suited for preparation of antibody liposome conjugates [72]. Chua et al used this approach to attach immunoglobulin to liposome surface [73]. In this study, carbohydrate groups in the heavy chain of the immunoglobulin were oxidation to afford aldehyde in the help of galactose oxidase or sodium periodate. Finally, immunoglobulin was immobilized onto the surface of liposome by formation of amide bonds between aldehyde in the immunoglobulin and hydrazide in the surface of liposome.

Additional amide bonds could be formed between p-nitrophenylcarbonyl group and primary amines (Figure 1.4 k). This method allows directly conjugation of antibodies into the liposome without additional modification. Torchilin et al successfully attached several proteins such as avidin, antimyosin antibody 2G4, wheat germ agglutinin and concanavalin into the liposome surface via a stable and non-toxic amide bond [74]. However, one main drawback of this approach lies in usage of bifunctional p-

nitrophenylcarbonyl because this bifunctional molecule offers a possibility of cross-linking.

Linkages containing thioethers

Considering thiol is another common group in the protein, the reaction of thioethers between protein and thiol-reactive liposome has been studied well for liposome surface modification. The thiol-reactive functional groups are kind of ankyllating reagents including include iodoacetamides (Figure 1.5A), maleimides (Figure 1.5B), and disulfides (Figure 1.5C). Iodoacetamides easily react with thiols group in the proteins peptides and antibodies via formation of thioethers. Normally, they are more likely reactive with thiol group in the cysteine but may also react with ones in the tyrosine or histidine. For instance, Frisch et al. has successfully attached peptides containing cysteine to the surface of liposome functionalized with bromoacetyl [75]. This coupling reaction was performed at pH 9.0 and done in one hour. The reaction condition developed here did not alter the integrity of liposome and are harmless to liposome surface modification.

The maleimide reaction is most effective and common approach in bioconjugation chemistry for surface modification due its high efficiency and selectivity with minimization of side reactions [Figure 1.5B]. This approach involves reaction of thiols in the proteins or peptides and double bond in the maleimides to form a stable thioether bond, allowing conjugation proteins or peptides covalently into liposome surface. A number of researches have been focused on liposome surface modification by maleimides reactions. For example, Garnier et al. recently immobilized the Annexin-A5 protein onto liposome surface modified with maleimides [76]. Another case is the selective

conjugation of thiolated OX26 monoclonal antibodies to DSPE-PEG-maleimide functionalized liposome [77].

Compared to iodoacetamides and maleimide reaction, disulfides reaction is freely reversible and thiol specific. The new disulfide bond was formed through a thiol-disulfide exchange and therefore improves resistant to hydrolysis. Gyongyossy-Issa et al used this approach to successfully conjugate RGD-motif-containing peptide into liposomes by a thiol-disulfide exchange and provides a choice for target drug delivery [78]

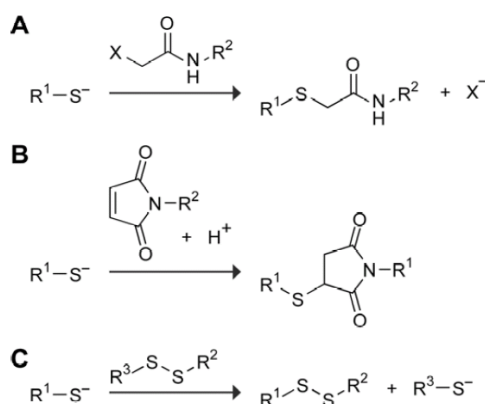


Figure 1.5 Bioconjugation via Thioethers or Disulfides. Reaction of a thiolate with (A) a haloacetamide, (B) a maleimide, and (C) a disulfide linkages. Reproduced from [79]

Containing carbon–nitrogen double Bonds

Bioconjugation via carbon–nitrogen double bonds by which ligands can be incorporated into liposome surface in physiological condition has received much attention. In this approach, nitrogen bases react with aldehydes and ketones to afford carbon-double bonds including hydrazones ($C=N-N$) and oximes ($C=N-O$). Nitrogen and Oxygen in the terminal of carbon-double bonds make it more stable compared to regular products of amines with aldehydes or ketones. A number of researches have been

conducted on liposome functionalization by antibodies or proteins by this strategy. In this strategy, aldehyde could be from ligands or performed liposome. Bonnet et al. successfully incorporate hydrazino-derivatized ligands to aldehyde functionalized liposomes via formation of hydrazone. In this study, functionalized anchor lipid (aldehyde functionalized ether lipid di-O-hexadecyl-rac-glyceraldehyde) was synthesized firstly and used for liposome preparation; next, lysosome-associated membrane protein (LAMP) was modified by N,N,N-tri(tert-butyloxycarbonyl)-hydrazino acetic acid and then incubated into aldehyde functionalized liposome to afford LAMP liposome conjugate. In contrast, Torchilin et al. chose amine functionalized liposome for conjugation of rabbit anticanine cardiac myosin antibodies [80]. In this experiment, glutaraldehyde and dimethyl suberimidate are utilized for conjugation via formation of oximes.

1.3.3.2 Bioorthogonal Conjugation

To date, most of available conjugation methods are non-specific and less selective interaction, and also suffer from low efficiency and toxicity to living organism. As a result, there are less reliable and safe for living systems. Fortunately, a number of bioorthogonal reactions have been developed. Compared to traditional chemical methods, these bioorthogonal reactions have shown excellent biocompatibility and selectivity in living systems. Each of bioorthogonal reactions used for liposome surface modification will be discussed in detail.

Native chemical ligation

Native chemical ligation is one of most widely used methods for chemical biology. This strategy was developed by Dawson et al to ligate large unprotected peptide fragments [81]. In this reaction, two unprotected peptide segments by the reaction of a α -thioester with a cysteine-peptide can be connected at the physiological condition (Figure 1.6). Numerous studies have been conducted on national chemical ligation and its applications such as protein synthesis, in vivo imagine, surface modification and protein array [82].

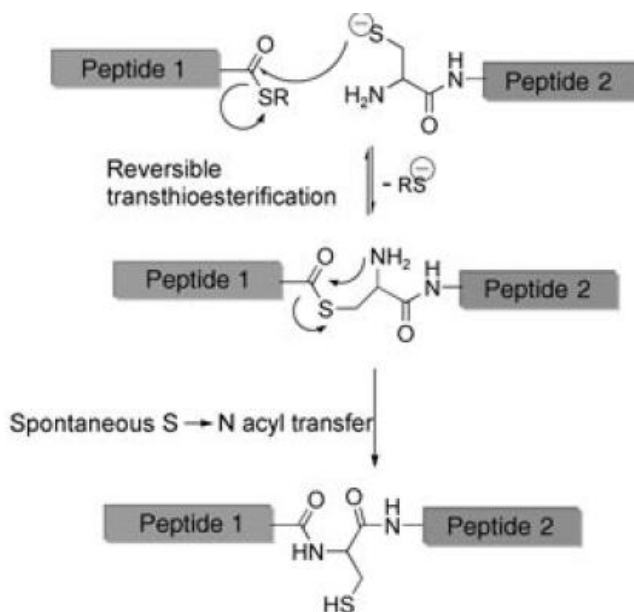


Figure 1.6 Principle of Native Chemical Ligation. Reproduced from [82]

Considering its chemoselectivity and high efficiency in physiological condition, Reulen et al. first introduced this ligation to liposome surface modification [83]. In this study, anchor lipid (DSPE-PEG-Cysteine) for native chemical ligation has been firstly synthesized and then was allowed to preparation of liposome containing anchor lipid. Next, Enhanced yellow fluorescent protein (EYFP) and a collagen-binding protein

domain (CNA35) modified with sodium 2-mercaptoethanesulfonate (MESNA) were chosen to perform native chemical ligation. After incubation in HEPES buffer for 48h, cysteine functionalized liposome has been successfully modified by EYFP and CNA35. The strategy developed here provides an alternative method for liposome surface modification.

Staudinger ligation

The Staudinger ligation, in which an azide and triphenylphosphine selectively react to form an amide, has been used for chemical selective modification of recombinant protein under native condition [84]. Recently, Staudinger ligation has been successfully performed in living animals without physiological harm, suggesting the potential for applications in cell surface functionalization [85]. Particularly, the reaction is known to occur in high yield at room temperature in aqueous solvents and without any catalyst, and is compatible with the unprotected functional groups of wide range of biomolecules. We have developed an efficient and chemically selective liposome surface functionalization with lactose as model carbohydrate through Staudinger ligation [38] (Figure 1.7). The high specificity and high yield as well as biocompatible reaction condition natures of the Staudinger ligation approach make it an attractive alternative to all current protocols for liposome surface functionalization.

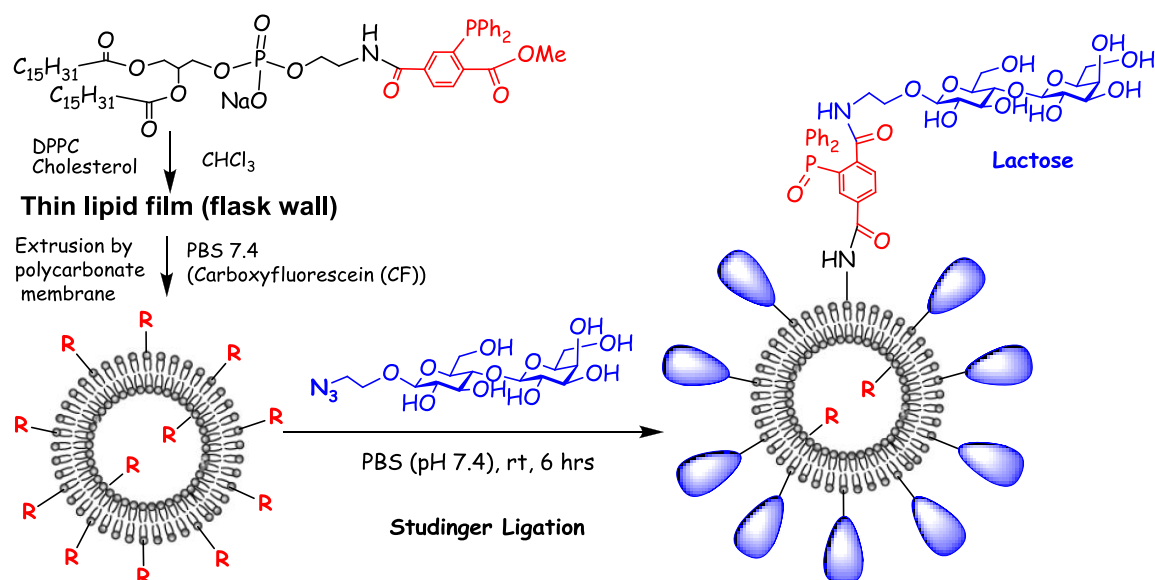


Figure 1.7 Schematic Illustration of Liposome Surface Glyco-Functionalization through Staudinger Ligation. Reproduced from [38]

Click chemistry

As one of the most popular reactions for bioconjugation techniques in chemical biology, Click chemistry was developed by K. Barry Sharpless based on the 1, 3-dipolar cycloaddition reaction between azides and alkynes to yield triazoles in 2001 [86]. In the reaction, an organic azide reacts with an alkyne to form a stable triazole ring under help of Cu (I) in aqueous conditions with high efficiency. Both of reactants-azide and alkyne are very inert and orthogonal to other chemical functionalities which offer this reaction high selectivity. Therefore, Click chemistry is extremely valuable in chemical biology by proving an excellent technology for bioconjugation and has attracted much attention in many areas such as protein modification, microarray, cell imaging and peptides synthesis. Hassane et al. successfully expanded this strategy into liposome surface functionalization [87]. First, anchor lipid N-[2-(2-(2-(2-(2,3-

bis(hexadecyloxy)propoxy)ethoxy)ethoxy)-ethoxy)ethyl]hex-5-ynamide was synthesized in which alkyne group was inserted into the terminal of lipid for conjugation. Then, liposome with incorporation of anchor lipid above were prepared and followed to incubate with an azido-modified mannose ligand for 24 h. After evaluation of mannose onto the surface of liposome, this in situ surface modification showed around 25% yield. Another example for liposome surface modification was adopted by Cavalli et al. [88]. In this study, Alkyne functionality was inserted into the liposome surface by incorporating alkyne functionalized 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). A single amino acid α -azido-modified lysine was chosen and mixed together with alkyne functionalized liposome to perform this coupling reaction under help of Cu (I) (Figure 1.8). Unfortunately, due to introduction of cooper as a catalyst, this strategy limited its wide application in living organism since cooper is heavy metal and could be toxic to living systems.

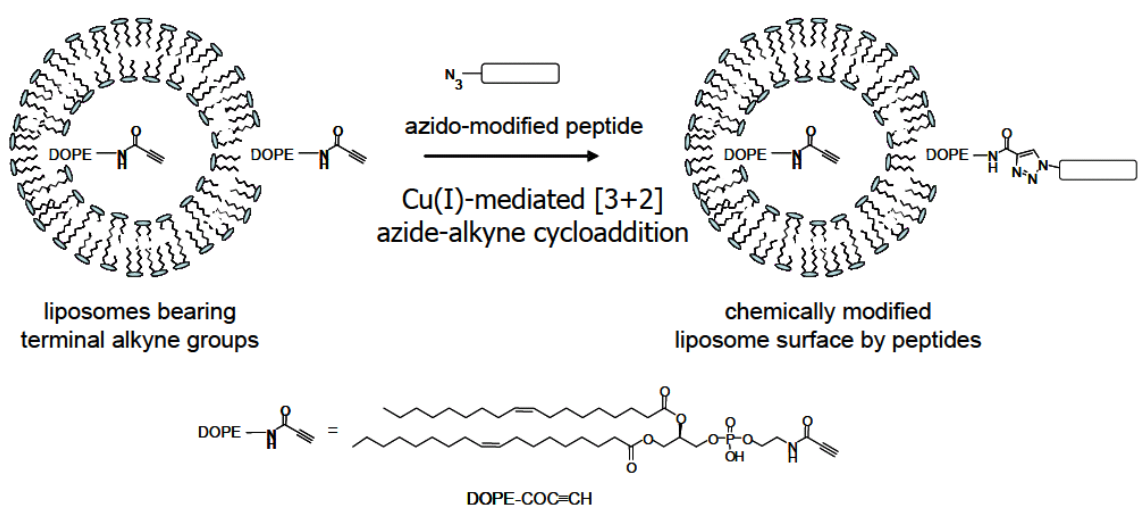


Figure 1.8 Schematic Representation of the General Approach for the Chemical Modification of a Liposome Surface by Peptides via Copper (I)-Mediated [3+2] Azide-Alkyne Cycloaddition. Reproduced from [88]

1.4 Incorporation of Unnatural Amino Acid for Protein Engineering through Synthetic Biology

Unnatural amino acids (UAAs), unlike a standard set of 20 amino acids, are not basic blocks for building living organism in the earth but still represent an enormous amount of diverse structural elements and provide a great potential to build a new protein with novel function or enhance properties to meet numerous needs of life. Sometimes UAAs other than basic amino acids plays a vital role in biology activity. For example, as an inhibitory neurotransmitter, gamma-amino butyric acid helps neurons stay selective about the signals to which they respond with a state of relaxation [89]. Histamine as an UAAs involves in local immune response and mediates allergic reactions [90]. On the other hand, UAAs lead to protein or even organisms with enhanced function. Schultz et al first introduced UAA into protein with providing an opportunity to examine the evolutionary consequences as well as generate proteins with new or enhanced biological functions [91]. The replacement of the natural L-amino acids with unnatural D-amino acids can make the peptides resistant to protect themselves from degradation by peptidases [92].

1.4.1 Unnatural Amino Acid Incorporation

The incorporation of UAAs into protein has attracted a lot of attention in chemical biology and pharmaceutical industry. This strategy not only aims to improve physiological properties but also create artificial proteins for pharmaceutical application. A variety of methods have been developed to explore the incorporation of UAAs into proteins. Herein, those methods for incorporation of UAAs have classified into three categories:

translational incorporation of UAAs, semi-synthetic incorporation and bio-orthogonal conjugation to UAAs and are described in the following.

1.4.1.1 Translational Incorporation of UAAs

The use of UAAs auxotrophic strains is the oldest method for UAAs incorporation by use of auxotrophic *Escherichia coli* (*E. Coli*) strains. In this strategy, incorporation of UAA into recombinant proteins exploits the unnatural substrate tolerance of the native translational apparatus to accept amino acids analogues. Therefore, two basic requirements should be met in the method. One is to prepare a set of specific amino acid analogs, another is to create a targeted amino acids auxotrophic strains. Tirrell et al successfully incorporated azido-homoalanine into protein (mDHFR) with high translational efficiency. In this experiment, Methionine-tRNA synthetases was used to accept several methionine analogs to incorporate into protein, only azido-homoalanine was recognized as a methionine surrogate and provided an opportunity to chemoselective modification and labeling of proteins in native conditions [84]. Except methionine, leucine [93], isoleucine [94], phenylalanine [95], tryptophan [96] and proline [97] auxotrophic strains have been investigated to incorporate UAAs into proteins. However, there are some basic issues to be addressed for ideal application. On one hand, the tolerance to analogs for an aminoacyl tRNA synthetase (aaRS) could be different and limited to further application. On the other hand, some amino analogs could be toxic to microorganisms and cause function loss of protein since they are faked to the living systems [98].

1.4.1.2 Incorporation of UAAs by Nonsense Suppressor tRNAs

Due to drawbacks in use of auxotrophic strains, UAAs incorporation into proteins by nonsense suppression has been used in attempts to replace a canonical amino acid with an analog and has proven to be a valuable tool for structure-function studies [99, 100]. In this strategy, the amber (UAG) nonsense codon was introduced to instead of the codon for the amino acid of interest. Since aaRS does not cross-react with any of the endogenous tRNAs or any different aminoacyl tRNA synthetase, this replacement will not recognize any of the common. As a result, desired amino acid will be recognized and insertion because of suppression of the nonsense codon with an aminoacylated tRNA [Figure 1.9]. Although it seems like a promising method to incorporate UAAs, its further application has been limited due to strict criteria to meet: the suppressor tRNA must have an efficient capacity to insert the UAAs and can not be recognized by any natural aaRS. Furthermore, only one stop codon can be used for suppression, incorporation of different UAAs into at one time becomes impossible [101].

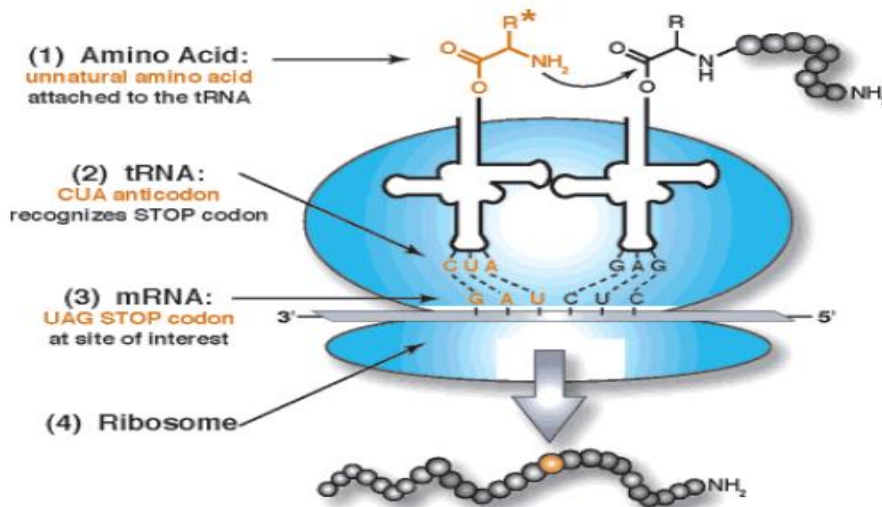


Figure 1.9 Incorporating Unnatural Amino Acids into Proteins Using Nonsense Codon Suppression. Reproduced from [102]

1.4.1.3 Incorporation of UAAs through Frameshift Suppression

To address those major disadvantages in the method of nonsense suppressor tRNAs, Sisido and coworkers have modified this method and successfully developed a remarkable approach for incorporation of UAAs, namely frameshift suppression, in which four-base codons have been created to assign non-natural amino acids within the framework of the existing three-base codon system.[103] Next, many more four-base codons such as pairs (AGGU, CGGU, CCCU, CUCU, and GGGU) have been examined and shown an high efficiency of 80% with respect to the wild-type protein [101]. Later, codons have even extended to five-base codons such as CGGN₁N₂, where N₁ and N₂ could be one of four nucleotides and further expand the pool of codons for incorporation of UAAs [104]. This strategy has been performed by using a unique tRNA/aaRS pair not only in *E. coli* but also in the eukaryotic cells such as the *Xenopus* oocytes [105]. Unfortunately, those efforts to perform frameshift suppression in *Xenopus* oocytes did not succeed because of poor suppression efficiency. To update, Dougherty et al appropriately designed two orthogonal frameshift suppressors (FS)-tRNAst two tRNAs with 4-base anticodons, in which both can deliver UAAs in response to the quadruplet codons CGGG and GGGU. As a result, suppression efficiency was overcome by successfully reducing incorporation of undesired UAAs into proteins in a *Xenopus* oocyte [106]. This expanding size of codons makes it possible to incorporate several different UAAs into proteins at one time.

1.4.1.4 Incorporation of UAAs by Creation of Unnatural Base Pairs

Creation of unnatural base pairs is another strategy for the extension of the genetic code. The most difficult challenge to expand size of codons is to synthesize novel unnatural base pairs that are orthogonal to the natural types. The first new base pair has been developed by Switzer et al. by creation of base pairs-isoC and isoG in 1989 [107]. The results confirmed that isoC and isoG base pair have been incorporated into DNA and that the protein has successfully expressed with an UAA in the help of a synthetic tRNA. Recently, a new non-natural named base-pyridine-2-one was developed by Hirao et al [108] and successfully incorporated into a mRNA in a DNA template. Several unnatural nucleotides base pairs with hydrophobicity has been created and used for incorporation of triphosphate [109]. However, in the strategy chemical reaction was used for synthesis of the mRNA and tRNA containing the non-natural bases; those unnatural base pairs must be orthogonal and be recognized by the DNA polymerase and RNA polymerase. Taken together, those factors above make it a long way to run before practical application.

1.4.2 Applications of Unnatural Amino Acid Incorporation

1.4.2.1 Identifying Structural and Functional Domains

Unnatural amino acid mutagenesis as a tool to investigate various structural and functional protein domains has been studied extensively. For instance, cation- π interaction, as an important noncovalent binding interaction relevant to structural biology has attracted much attention [110, 111]. Particularly, cation- π interactions play a vital role in biological recognition, especially in the binding of acetylcholine biology. Zhong et al. explored this function by incorporating serial fluorine substitutions in Trp-149 of the muscle type nAChR via unnatural amino acid mutagenesis [112]. In this research, a

cation- π interaction between acetylcholine, a quarternary amine, with the ligand binding domain of the nAChR was confirmed and pointed out electrostatic potential of the tryptophan indole ring. Since lots of protein are supposed to have cation- cation- Π interaction, incorporation of unnatural amino acid could be very useful tool to explore this interactions. Also, the strategy of incorporation of UAAs has proven to be a powerful tool for investigating protein dynamics. For example, the incorporation of fluorescent side chains in an UAAs by using the nonsense suppression approach can monitor protein conformational change [100, 113]. Furthermore, two kinds of fluorescent side chains can be incorporated into one protein and provide an opportunity to explore cleavage of protein [114]. Additionally, the role of individual hydrogen bonds in protein stability and activity has been explored by incorporation of UAAs into proteins. A number of unnatural amino acids and amino acid analogs were synthesized and replaced alanine-82 in T4 lysozyme to determine the effect of replacement on the stability of T4 lysozyme. The replacements of alpha, alpha-disubstituted amino acids, N-alkyl amino acids removed a hydrogen bond donor at those sites and reduced its stability and activity [115].

1.4.2.2 Inserting Functionalities into Proteins

Incorporation of UAAs into proteins provides a powerful tool to engineer proteins with additional functionalities for special applications, especially for those functionalities that cannot be introduced translationally into large, interesting proteins. For instance, p-carboxymethyl-L-phenylalanine can be incorporated into Tyr701 in human signal transducer and activator of transcription-1 (STAT1) instead of phosphotyrosine to improve resistant to hydrolysis by tyrosine phosphatases [116]. This directly introduce

modifications in proteins provides a certification for protein engineering to expand functions. Also, incorporation of UAAs into proteins has numerous applications in protein tagging and protein conjugation. Several kinds of methionine analogs containing azides into recombinant proteins for chemoselective modification have been incorporated into murine dihydrofolate reductase (mDHFR) [102]. This incorporation of UAAs containing azides provides very useful tool for a lots of application such as bioconjugation, in vivo imaging, protein labeling or drug design and delivery by Staudinger ligation and Click chemistry. For instance, site-specific PEGylation of human thrombomodulin has been performed to increase half-time in the blood via Staudinger ligation. [117]. An engineered LpIA acceptor peptide (LAP) with an alkyl azide have been developed and further labeled in living mammalian cells [118]. This strategy developed here provides an opportunity to biochemical and imaging studies of cell surface proteins.

1.4.2.3 Studying Signal Transduction

Incorporation of UAAs into proteins is becoming a powerful tool for investigating signal transduction pathways. A wide range of researches in vitro translation systems has been performed to explore and understand signal transductions in E.coli or yeast express systems by incorporation of UAAs. The protein phosphorylation is essential in regulating signal transduction cascades associated with cancer and other biological processes. Vázquez et al [119] studied phosphorylation dependent signaling by a series of phosphorylated residues prepared via incorporation of sensitive fluorescent amino acid DANA in the sequence RLYRpSLPA. The control of phosphorylation-dependent signaling has been achieved by the development of phosphorylated residues and provided

a useful tool to explore the role of protein phosphorylation in signal transduction cascades.

1.4 Research Design and Rational

Thromboembolic diseases such as myocardial infarction, stroke, and thromboembolism are severe with significant mortality and morbidity in the United States. Antithrombotic agents that prevent blood clotting can be used for both the prevention and treatment of active vascular thrombosis. However, current antithrombotic therapy is limited by the risk of series bleeding, hemorrhagic complication. A direct correlation exists between the intensity of anticoagulation and severity of bleeding. Recent advances in the molecular bases of haemostasis have highlighted new targets for novel antithrombotic agent design. Specifically, endothelial thrombomodulin (TM) plays a critical role in local haemostasis by binding thrombin and subsequently converting protein C to its active form (APC. In addition, the binding of thrombin to TM drastically alters the thrombin's procoagulant activities to anticoagulant activities. Importantly, TM expression, however, decreases in perturbed endothelial cells, predisposing to thrombotic occlusion and particularly in response to a variety of inflammatory stimuli, direct vessel wall injury, and oxidant stress. TM is a type I membrane protein. The lipid bilayer in which it resides serves as an essential 'cofactor', locally concentrating and coordinating the appropriate alignment of reacting cofactors and substrates for protein C activation. Lipid membranes also play pivotal roles in activation of other anticoagulant enzymes. On the other hand, liposomes have been extensively studied as cell surface model as well as carrier for delivering certain vaccines, enzymes, drugs, or genes to the body. In this

study, we hypothesized that combination of antithrombotic membrane protein TM into lipid membrane mimetic assembly (liposome) through recombinant and bioorthogonal conjugation techniques provides a rational strategy for generating novel and potential antithrombotic agent (as shown in Figure 1.10). Namely, the liposomal TM conjugate mimics the native endothelial antithrombotic mechanism of both TM and lipid components and thus is more forceful than current antithrombotic agent.

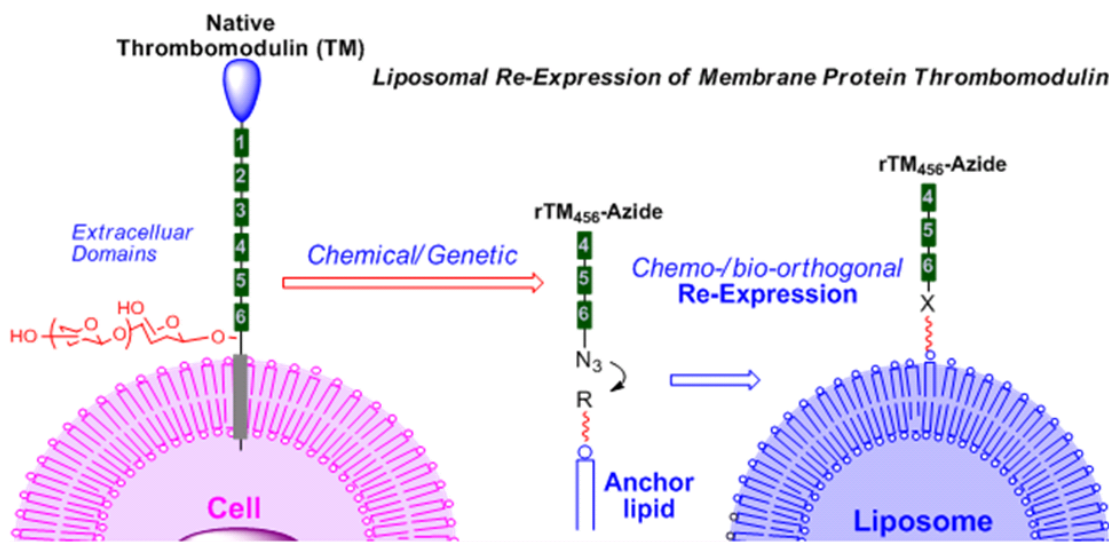


Figure 1.10 Schematic Illustration of Proposed Membrane Mimetic Re-expression of Membrane Protein onto Liposome.

1.5 References

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CHAPTER II

SURFACE FUNCTIONALIZATION OF LIPOSOME THROUGH STAUDINGER LIGATION

(Partial results from Zhang, H. L., Ma, Y., Sun, X-L. Chem. Comm. 2009, 3032-3034)

2.1 Introduction

Liposome, a spherical closed self-assembled (phospho) lipids, has been extensively studied as model of cell membrane and mostly as carrier for drug/gene delivery applications [1, 2]. Liposome surface functionalization facilitates enormous potential application of liposomes [3, 4]. Cell surface carbohydrates is attractive model for liposome surface modification with oligosaccharides due to their protein-rejecting ability, biodegradability, low toxicity, and especially, cell targeting ability through specific binding interactions with receptors expressed at the targeted cells surface. For example, monosialoganglioside (GM1) can enhance circulation lifetimes of liposomes comparable to that observed for PEG [5]. Sialyl lewis X decorated liposome has been demonstrated to target delivery of drugs to endothelial cells based on the site-specific expression of E- and P-selectin on the blood vessel during inflammation [6]. On the other

hand, carbohydrate-decorated liposomes have been used as multivalent platform of carbohydrates to inhibit carbohydrate-mediated cell adhesion. For example, sialic acid-decorated liposome showed strong inhibitory activity against influenza hemagglutinin and neuraminidase.

Conventional method to prepare surface functionalized liposome involves the initial synthesis of the key lipid-ligand conjugate, followed by formulation of the liposome with all lipid components. In this direct liposome formation method, some of the valuable ligands inevitably are facing the enclosed aqueous compartment and thus become unavailable for their intended interaction with their target molecules. Particularly, it is unrealistic if the targeting ligand is only available in minimum amount. Furthermore, lipid-ligand conjugates normally have limited solubility and stability in solvent, or are incompatible with various stages of manufacture. Alternatively, chemical modification methods, which in most cases involve the coupling of biomolecules to the surface of preformed vesicles that carry functionalized (phospho)lipid anchors, have been developed [8,9]. So far, variable success using amide [10] or thiol-maleimide coupling [11] as well as by imine [12] or hydrazone linkage [13] have been achieved. However, in many cases there is a lack of specificity resulting in the uncontrolled formation of the number of covalent bonds between liposome and biomolecules of interest. Most recently, copper (I)-catalyzed [3+2] cycloaddition, namely “Click” chemistry, which can occur efficiently in aqueous media at room temperature and selectively between azide and alkyne [14] has been investigated as novel generic chemical tool for the facile in situ surface modification of liposomes [15]. However, the key limitation of the Click chemistry is the use of Cu (I) catalyst, which results in residual copper in the targeted liposome preparations and could

be a potential concern. Normally, it may be difficult to completely remove copper out from the resultant liposome [15] and thus it will affect the liposome's biological application later on.

The Staudinger ligation, in which an azide and triphenylphosphine selectively react to form an amide has been used for chemical selective modification of recombinant protein under native condition [16]. Recently, Staudinger ligation has been successfully performed in living animals without physiological harm, suggesting the potential for applications in cell surface functionalization [17]. Particularly, the reaction is known to occur in high yield at room temperature in aqueous solvents and without any catalyst, and is compatible with the unprotected functional groups of wide range of biomolecules. Herein, we report an efficient and chemical selective liposome surface functionalization with lactose as model carbohydrate through Staudinger ligation (Figure 2.1). The high specificity and high yield as well as biocompatible reaction condition natures of the Staudinger ligation approach make it an attractive alternative to all current protocols for liposome surface functionalization.

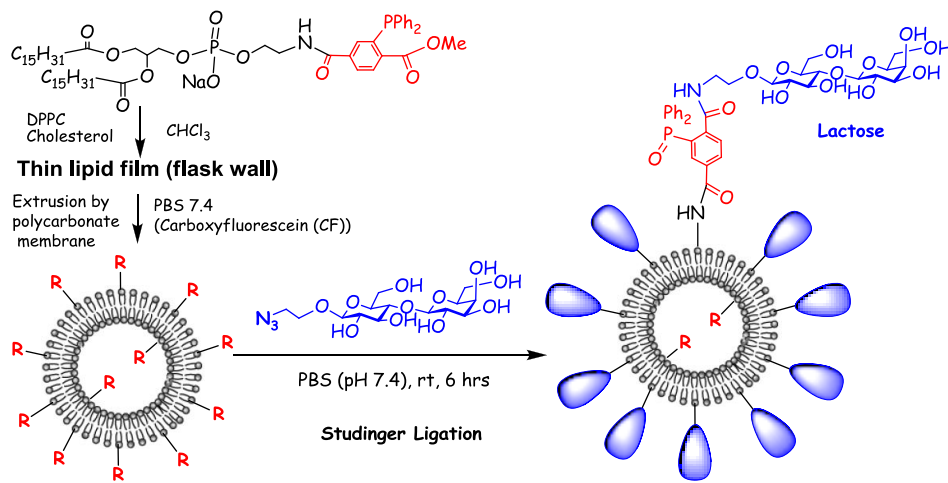


Figure 2.1 Schematic Illustration of Liposome Surface Glyco-Functionalization through Staudinger Ligation.

2.2. Experimental

2.2.1 Materials and Methods

All solvents and reagents were purchased from commercial sources and were used as received, unless otherwise noted. Deionized water with a resistivity of 18 Ω was used as a solvent in all polymerization reactions.

Thin-layer chromatography (TLC) was performed on Whatman silica gel aluminum backed plates of 250 nm thicknesses on which spots were visualized with UV light or by charring the plate after dipping in 10 % H_2SO_4 in methanol. ^1H NMR spectra were recorded at room temperature with a Varian INOVA 300 MHz spectrometer. In all cases, the sample concentration was 10 mg/mL, and the appropriate deuterated solvent was used as an internal standard. Dynamic Light Scattering was recorded with 90Plus particle size analyzer (BIC). Fluorescent spectrum was measured with FluoroMax-2 (ISA)

2.2.2 Synthesis of DPPE-triphenylphosphine for Chemoselective Surface Functionalization via Staudinger Ligation

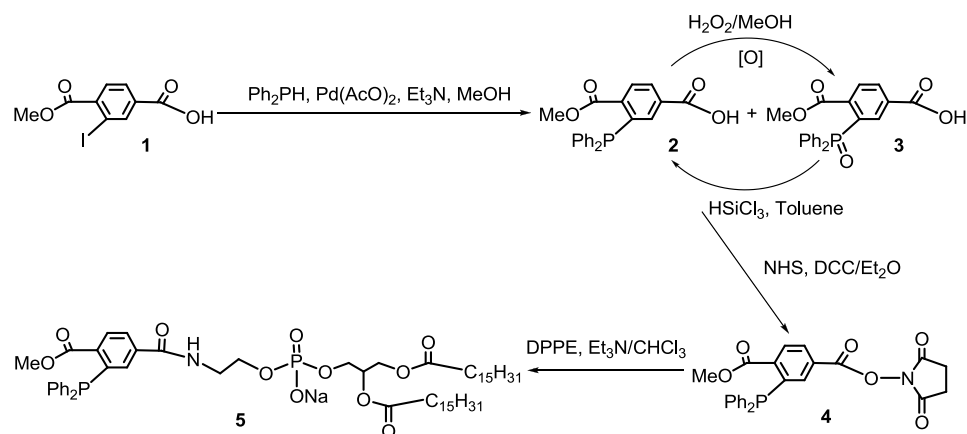


Figure 2.2 Scheme of Synthesis of DPPE-Triphenylphosphine

2.2.2.1 Synthesis of 1-Methyl-2-Diphenylphosphinoterephthalate

1-Methyl-2-Diphenylphosphinoterephthalate (2) Dry MeOH (3ml), triethylamine (TEA) (0.3 ml, 2 mmol), compound 1 (300 mg, 1.00 mmol), and palladium acetate (2.2 mg, 0.010 mmol) were added to a flame-dried flask. The mixture was degassed in vacuo. While stirring under an atmosphere of Ar, and then diphenylphosphine (0.17 mL, 1.0 mmol) was added to the flask with a syringe. The resulting solution was heated at reflux overnight, and then allowed to cool to room temperature and concentrated. The residue was dissolved in 250 mL of a 1:1 mixture of CH₂Cl₂/H₂O and the layers were separated. The organic layer was washed with 1 M HCl (10 mL) and concentrated. The crude product was dissolved in a minimum amount of methanol and an equal amount of H₂O was added. The solution was cooled to 4 °C for 2 hrs and the resulting solid was collected by filtration. The pure product 3 was isolated as 245 mg (69 %) of a golden yellow solid, mp 183–185°C. ¹H NMR (400 MHz, CDCl₃): 3.75 (s, 3H), 7.28–7.35 (m, 11H), 7.63–7.67 (m, 1H), 8.04–8.07 (m, 1H).

2.2.2.2 Synthesis of 1-Succinimidyl 3-Diphenylphosphino-4-Methoxycarbonylbenzoate

1-succinimidyl 3-diphenylphosphino-4-methoxycarbonylbenzoate (4) 3, 3-diphenylphosphino-4-methoxycarbonylbenzoic acid (216mg, 0.593mmol), dicyclohexylcarbodiimide (DCC) (129mg, 0.623mmol), and N-hydroxysuccinimide (NHS) (72 mg, 0.623 mmol) were dissolved in diethyl ether (20 mL) under Ar and stirred overnight at room temperature. After the diethyl ether solvent was evaporated, the yellow crystalline product was isolated by filtration. Recrystallization from 2-propanol (20 mL) yielded 164 mg (60 %) of 3 as yellow crystals. ^1H NMR (CDCl_3 , 400 MHz) (ppm): 8.15 (s, 2H), 7.67 (d, 1H, J), 7.39-7.22(m, 10H), 3.75 (s, 3H), 2.82 (s, 4H, $\text{COCH}_2\text{CH}_2\text{CO}$); ^{13}C NMR (CDCl_3 , 75 MHz) δ 169.4 ($\text{COCH}_2\text{CH}_2\text{CO}$), 166.7, 161.5, 143.0, 142.6, 140.1, 139.9, 137.0, 136.8, 136.6, 134.5, 134.2, 131.3, 130.2, 129.6, 129.2, 129.1, 128.1, 53.0, 26.0 ($\text{COCH}_2\text{CH}_2\text{CO}$); ^{31}P NMR (CDCl_3 , 121 MHz), δ (ppm): -3.05

2.2.2.3 Synthesis of DPPE-Triphenylphosphine

DPPE (0.26 g, 0.376 mmol) was dissolved in 40 mL CH_2Cl_2 , and 0.8 mL Et_3N was added. After stirring for 30 mins at room temperature, a solution of Succinimidyl 3-Diphenylphosphino-4-methoxycarbonylbenzoate (4) (0.23 g, 0.500 mmol) in 50 mL CH_2Cl_2 was added. The reaction mixture was stirred at room temperature for 24 hrs and then concentrated. The crude product was purified by TLC using chloroform/methanol (4: 1) to afford 5 (0.283g, 73%). ^1H NMR (CDCl_3 , 400 MHz) δ (ppm): ^{31}P NMR (CDCl_3 , 121 MHz), δ (ppm)

2.2.3 Preparation of Unilamellar Liposome for Surface Functionalization

DPPE (30mg, 40.87 μmol), cholesterol (8mg, 20.4 μmol), DPPE-triphenylphosphine (3.5 mg, 3.2 μmol) (2: 1: 5 % molar ratio) were dissolved in 3.0 mL chloroform. The lipid mixture was dried onto the wall of a 50 mL round-bottom flask and the solvent was gently removed on an evaporator under reduced pressure to form a thin lipid film on the flask wall and kept in a vacuum chamber overnight. Then, the lipid film was swelled in the dark with 2.5 mL PBS buffer (pH 7.4) containing 85 mM Carboxyfluorescein (CF) to form the multilamellar vesicle suspension. Followed to be subjected 10 freeze-thaw cycles involving quenching in liquid N_2 and then immersed in a 50 $^{\circ}\text{C}$ water-bath. The crude lipid suspension thus formed, followed by extruded through polycarbonate membranes (pore size 2000 nm, 600 nm, 200 nm, 100 nm gradually) to obtain the vesicles (LUVs) with approximately uniform diameter (110 ± 5 nm). Separation of the CF vesicles from non-entrapped CF was achieved by gel chromatography, which involved passage through a 1.5 \times 20 cm Column of Sephadex G-50), about 6ml liposome were gained.

2.2.4 Lactose Coupling to Performed Liposome via Staudinger Ligation

To the liposome made in the part of preparation, added 4 mg azido-lactose in 0.2 mL PBS buffer (Argon bubble before use) into 2 mL liposome. The Staudinger ligation was run at room temperature for 6h under an argon atmosphere, then the unreacted azido-lactose was removed by gel filtration (1.5 \times 20 cm Column of Sephadex G-50). The

stability of liposome was monitored by FluoroMax-2 (ISA). The size of liposomes in the Staudinger ligation was monitored over time by using 90Plus particle analyzer.

2.2.5 Determination of Lactose Density on the Surface of Liposome

The liposomes (5 mg/mL, total lipid concentration) separated from the column was divided into two parts, one part was for reaction with lactose-azide, another was for control.

Assay: 20 μ L reaction solution and 1980 μ L PBS were mixed, and then diluted into 10 times.

Control: 20 μ L original solution (5 mg/mL) and 1980 μ L PBS were mixed and then diluted into 10 times.

Standard curves of lactose assay: The method is that described by Dubois et al. with some modifications. To 0.5 ml of lactose solution (20 μ g/mL, 40 μ g/mL, 60 μ g/mL, 80 μ g/mL, 100 μ g/mL, respectively), 0.5ml of 5% phenol solution was added and mixed. Then 2.5 mL concentrated H_2SO_4 was added directly into the solution with 1-2 s. The mixture was then vortexed, and allowed to stand for 30 mins at room temperature. Readings were taken at 490 nm against a blank prepared by substituting distilled water for the lactose solution (Figure 2.3).

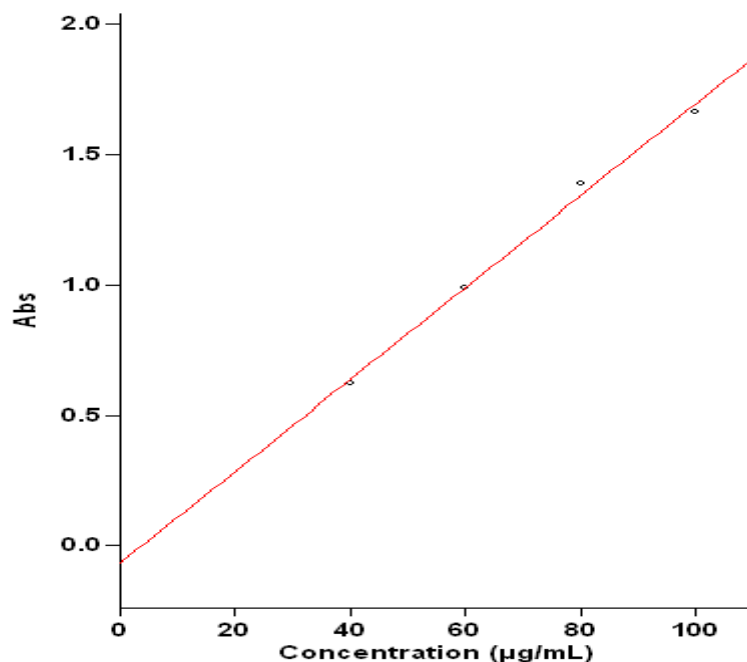


Figure 2.3 Standard Curves of Lactose Assay

Colorimetric analysis: To 0.5 mL of liposome with immobilized lactose, 0.5ml of 0.5 % phenol solution was added and mixed. Then 2.5 mL concentrated H_2SO_4 was added directly into the solution with 1-2 s. The mixture was then vortexed, and allowed to stand for 30 mins at room temperature. Readings were taken at 490 nm. The amount of lactose from calibration curve was calculated.

2.2.6 Assay for Coupling Accessibility of Functionalized Liposome

To detect the lactose in the liposome, 0.5 mL PBS buffer (pH 7.4) including 0.5 mg lectin was added into 100 µL liposome conjugating the lactose. In the reaction, the size of liposomes and stability of liposome in the reaction were monitored over time (0h, 1 h, 2 h, 3 h, 4 h), and a control was performed at the same conditions, too.

2.2.7 Evaluation of Stability of Functionalized Liposome by Dynamic Light Scattering and Florescence Assay

The stability of liposome was also monitored by measurement of fluorescent leakage using FluoroMax-2 (ISA). 20 μ L reaction solution and 1980 μ L PBS were mixed, and then diluted into 10 times. The fluorescent intensity was measured by using FluoroMax-2 (ISA). Control experiment was conducted in the absence of azide-lactose.

5, 6-carboxyfluorescein (CF) released from liposomes in PBS (pH 7.4) buffer at room temperature was measured over time. The excitation and emission wavelengths of 5, 6-CF were 497 and 520 nm, respectively. The variation of the fluorescent intensity with release time was calculated according to the equation below

$$\text{Fraction of CF remaining in liposomes} = 1 - F/F_0$$

where F is the fluorescent intensity measured at any time during the experiment and F_0 is the total fluorescent intensity measured after disrupting liposomes completely with 0.5% Triton X-100 in PBS (pH 7.4) buffer.

Liposome size and distribution were analyzed by dynamic/static light scattering with 90Plus particle size analyzer (BIC).

2.3 Results and Discussion

2.3.1 Characterization of DPPE-Triphenylphosphine

As a proof-of-concept, we have explored the possibility to prepare glycosylated liposomes by coupling unprotected lactosyl derivative carrying an ethyl spacer

functionalized with an azide group to the surface of liposomes that incorporate synthetic anchor lipids carrying a terminal triphenylphosphine via Staudinger ligation. First, the terminal triphenylphosphine carrying anchor lipid 5 was synthesized by amidation of commercially available DPPE with 3-diphenylphosphino-4-methoxycarbonylbenzoic acid NHS active ester 4, which was prepared according to the procedure described by Ju et al. [18] (Figure 2.4). However, it was found that an unwanted oxidation product, triphenylphosphine oxide 3 easily formed during crystallization purification of triphenylphosphine 2 by using aqueous methanol. The oxidized compound 3 was confirmed by comparing with oxidation product of triphenylphosphine 2 with hydroperoxide in methanol, which give a typical chemical shift at 34.2 ppm of phosphine oxide, while the phosphine in 2 gives a chemical shift at -3.74 ppm in ^{31}P NMR spectrum (Figure 2.3A). Fortunately, the phosphine oxide 3 can be converted back to phosphine 2 by reduction with trichlorosilane in toluene in high yield [19]. There is no oxidized product formed during DPPE-triphenylphosphine 5 synthesis and purification when organic solvents are used. With this oxidation in mind, the stability of the intermediate triphenylphosphine 2 and the targeted lipid triphenylphosphine conjugate 5 were monitored with ^{31}P NMR. As results, the oxidation of phosphine 2 and 5 in organic solvent such as chloroform are very slow (Figure 2.4B and 2.4C). Mass Spectrometry has been used to confirm its molecule weight (1036) (Figure 2.4D)

A

B

C

D

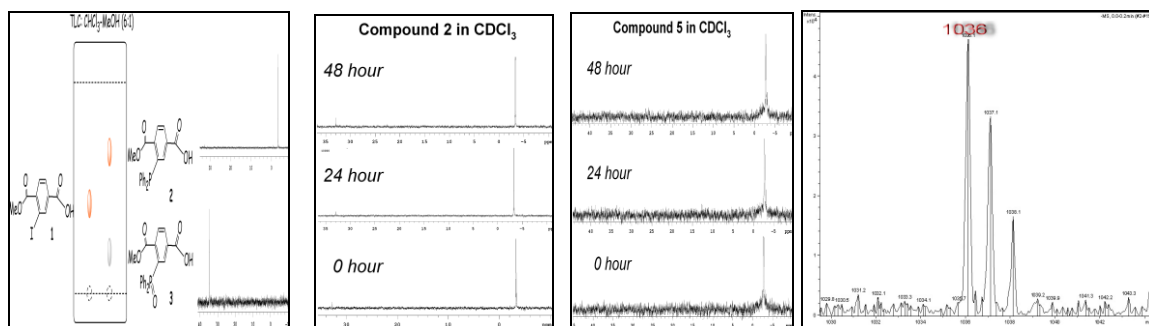


Figure 2.4 TLC traces and ^{31}P NMR Study of Triphenylphosphine Derivatives

2.3.2 Integrity Assay of Liposome in and after Coupling Reaction

With the anchor lipid DPPE-triphenylphosphine 5 in hand, next, small unilamellar liposomes composed of saturated phospholipids (DPPC) and cholesterol (2: 1 molar ratio) and 5 mol % of the anchor lipid 5 were prepared by extrusion through polycarbonate membranes with pore size of 600 nm, 200 nm, and 100 nm sequentially at 65 °C. This produced predominately small unilamellar vesicles with an average mean diameter of 120 ± 5 nm as determined by dynamic light scattering (DLS). Next, conjugation of azide-containing lactose ligands [20] to the preformed liposomes was performed in PBS buffer (pH 7.4) at room temperature under an argon atmosphere for 6 hrs (Figure 2.1). DLS was used to verify the integrity of the vesicles during and after the coupling reaction. As shown in Figure 2.5, there is no significant change in the size of the vesicles during and after conjugation reaction. Therefore, the reaction conditions described above do not alter the integrity of the liposomes.

A

B

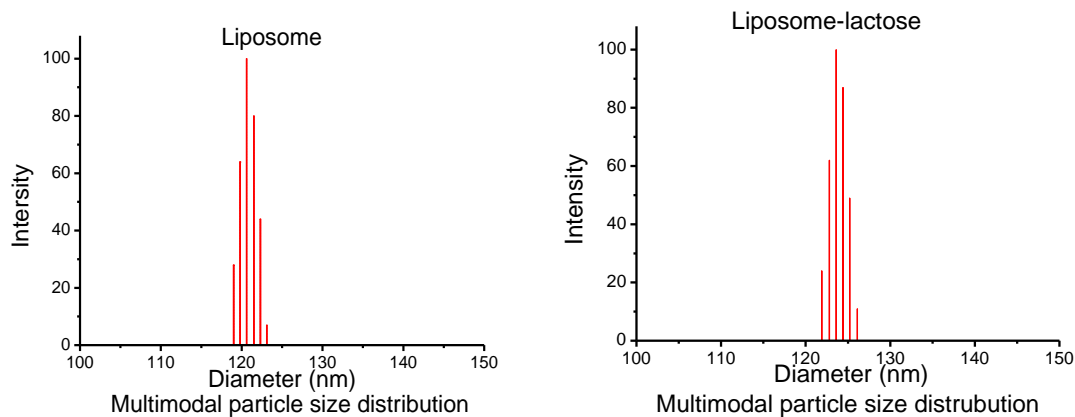


Figure 2.5 Dynamic light scattering monitoring of liposome, A: before conjugation, B: after conjugation

Next, to test whether the experimental conditions used for the conjugation reaction could provoke some leakage of the liposomes, we have exposed our standard conditions to the same type of liposomes having encapsulated self-quenching concentrations of 5, 6-carboxyfluorescein [21]. Based on the fluorescence quenching determinations (Figure 2.6), we could demonstrate that no apparent leakage was triggered by the conjugation reaction compared to the liposomes incubated in the absence of the coupling reagents. Therefore, the conjugation conditions established here are harmless for liposome surface functionalization.

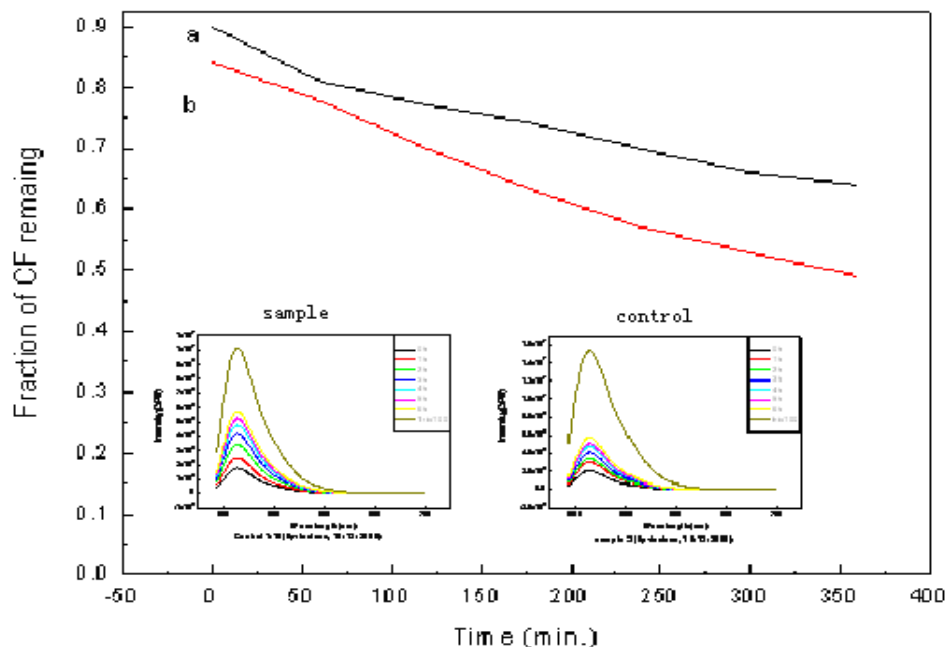


Figure 2.6 Kinetics of carboxyfluorescein release from liposome in the presence of lactose-azide (A) and in the absence of lactose-azide (control experiment) (B). Insert shows the changes of fluorescence intensity of liposomes suspension of reaction and control during conjugation and final decomposition with surfactant.

2.3.3 Quantification of Lactose on the Surface of Liposome

Furthermore, the grafted carbohydrate on the surface of liposome was quantified by the phenol-sulfuric acid method [22]. Briefly, phenol solution was added to a solution of liposome with conjugated lactose, and mixed. Then concentrated H_2SO_4 was added directly into the solution. The mixture was then vortexed, and allowed to stand for 30 mins at room temperature. The optical density was then recorded at 490 nm. Considering that about 40 % of the triphenylphosphine anchor is oriented toward the interior of the vesicles, 80 % of the out-oriented triphenylphosphine have been modified in this Staudinger ligation modification.

2.3.4 Assay for Coupling Accessibility of Functionalized Liposome

To determine whether the grafted lactose residues are easily accessible at the surface of liposomes, lectin binding assay was conducted by incubating lactosylated liposome in the presence of galactose binding lectin (*Arachis hypogae*, 120 kDa, Sigma) in PBS (pH 7.4). After 30 mins, apparent visible aggregation formed and was monitored in DLS experiment (Figure 2.7A). In contrast, neither aggregation nor size change was observed with control liposomes without lactose. Furthermore, the presence of free lactose (5.0 mM) prevented aggregates formation (not shown), confirming that the agglutination was due to a specific recognition of the lactose residues on the surface of the liposomes by lectin in multivalent interactions (Figure 2.7B).

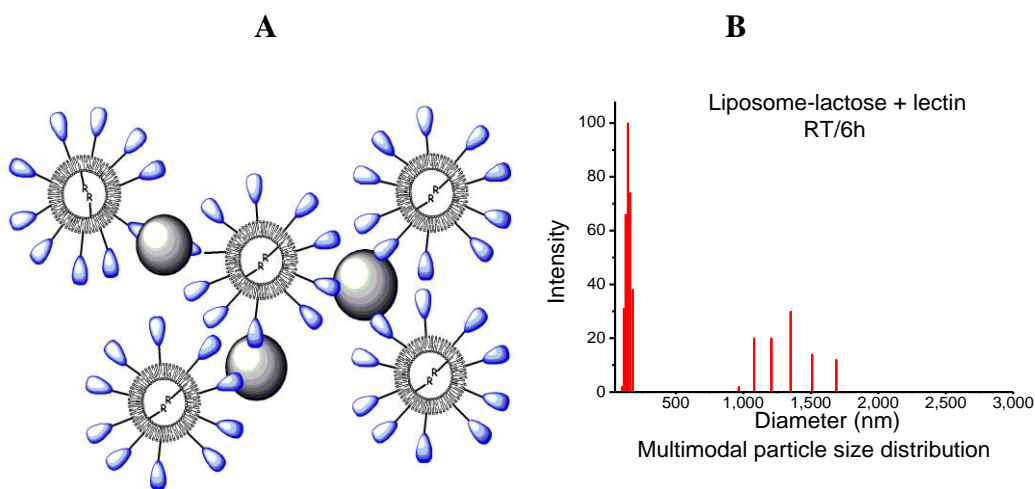


Figure 2.7 Agglutination glycosylated liposome with lectin via multivalent interaction (A) and monitored with DLS (B)

2.3.5 Evaluation of Stability of Functionalized Liposome

The stability of liposomes over time is an important concern in drug/gene delivery application. It is known that monosialoganglioside (GM1) could enhance circulation lifetimes of liposomes comparable to that observed for PEG. In this study, the stability of the lactosylated liposome was evaluated by comparing with liposome without lactose at room temperature as monitored with DLS. As shown in Figure 5, both liposomes showed good stability during the eight days period. However, the liposome without lactose began to collapse and aggregate since 9th day (Figure 2.8A), while there were no apparent size change for the lactosylated liposome (Figure 2.8B). This result demonstrated that the presence of lactose on the liposome surface provides a steric barrier that prevents liposome aggregation.

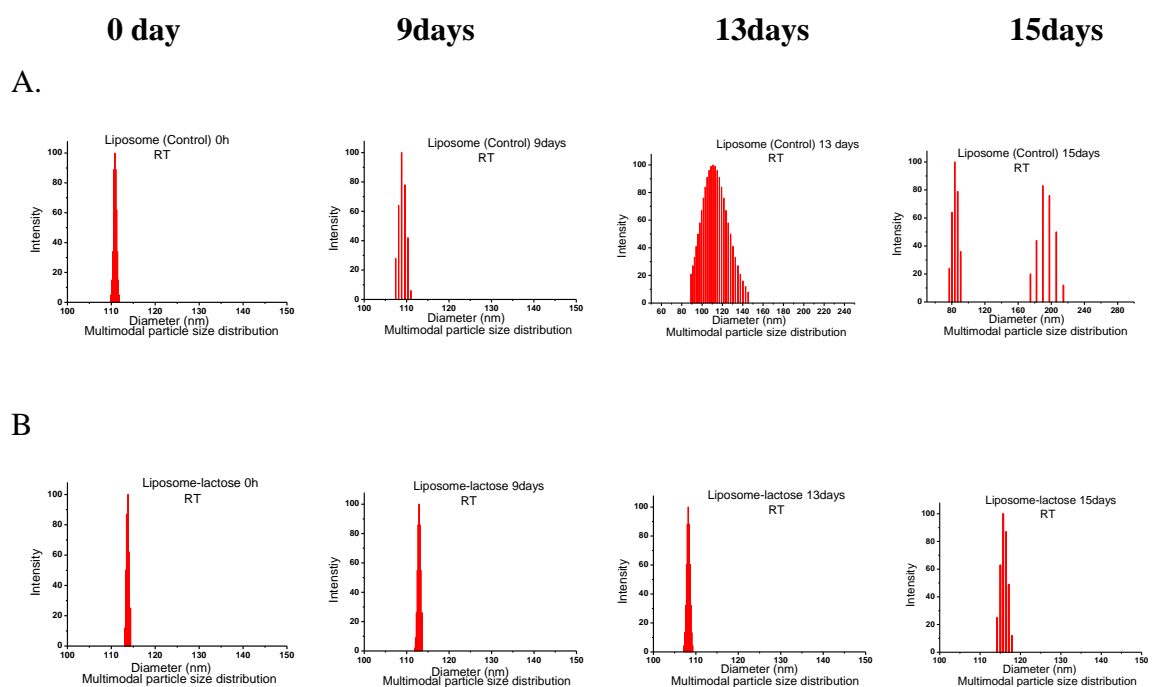


Figure 2.8 Stability of liposome without lactose (A) and liposome with lactose (B) monitored with DLS

2.4 Conclusion

In conclusion, we have developed an efficient and chemoselective conjugation method for liposomes surface glyco-functionalization based on Satudinger ligation. The reaction could be performed under mild conditions in aqueous buffers without catalyst and in high yields under reasonable reaction times. The reaction conditions developed in the present work did not alter the integrity of the bilayers, in terms of liposome sizes and leakiness, and provided perfectly functional vesicles. This versatile approach, which is particularly suitable for the ligation of water soluble molecules and which can accommodate many chemical functions, is anticipated to be useful in the coupling of many other ligands onto liposome.

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CHAPTER III

INCORPORATION OF UNNATURAL HOMOANALINE INTO

RECOMBANANT THROMBOMODULIN FOR SITE SPECIFIC CONJUGATION

(Protocol modified from Cazalis, C. S., Haller, C. A., Sease-Cargo, L., Chaikof, E. L.
Bioconjug. Chem. 2004, 15, 1005–1009)

3.1 Introduction

Thrombomodulin (TM) is an endothelial anticoagulant protein that contributes to local hemostatic balance by modulating the activity of thrombin from a procoagulant to an anticoagulant protease [1–3]. Through binding with thrombin to form complex on the endothelial surface, TM removed thrombin's procoagulant properties by stopping cleavage of fibrinogen or activation of platelets. At the same time, the TM-thrombin complex involves in protein C activation pathway in which protein C is bound to TM-thrombin complex to immediately enhance activation over 1,000 fold. Subsequently, the activated form of protein C (APC), an anticoagulant protease, selectively inactivates coagulation factors Va and VIIIa, providing an essential feedback mechanism in preventing excessive coagulation. TM has been considered as one of key factors contributing to thrombotic regulation [4]. It also has been reported that reduction in TM

activity, induced by a targeted point mutation or injection of anti-TM antibodies in mice, makes the animals more susceptible to thrombogenic challenges [5, 6]. Administration of exogenous recombinant TM to these animals has shown antithrombotic effects. In the past decades, various recombinant TM proteins consisting of extracellular portions have shown potential anticoagulant activity [7–12]. One such example is recombinant human soluble TM α (rhsTM α or ART-123), which was used in Japan to treat patients with disseminated intravascular coagulation (DIC) [12, 13].

The structure of TM and its structural domains necessary for activation of protein C have been resolved. The fifth and sixth EGF domains of TM represent the binding site of thrombin, while the fourth EGF domain is responsible for protein C activation, all which are related to TM's anticoagulant activity [2, 3]. However, TM's third EGF-like domain is of equal importance in the thrombotic cascade accelerating the proteolytic activation of thrombin-activatable fibrinolysis inhibitor (TAFI) by thrombin [14]. Activated TAFI (TAFIa) cleaves C-terminal lysine and arginine residues from partially degraded fibrin. The removal of these positively charged residues suppresses the ability of fibrin to catalyze plasminogen activation and thereby delays clot lysis [15]. Therefore, recombinant TM containing all extra cellular portions of TM simultaneously functions as a procoagulant and anticoagulant which would be reasonably ineffective as an anticoagulant therapeutic. Consequently, recombinant TM containing the EGF-like domains 456 (rTM₄₅₆) is the only engineered TM recombinant considered to be a potential candidate as a pure anticoagulant [16–19]. The smaller protein fragment, from a bioengineering standpoint, provides an opportunity through manipulation and modification to optimize and generate a feasible biotherapeutic.

Incorporation of unnatural amino acids to proteins has become a very popular tool to expand protein's functions and shown its promising potential in chemical biology and pharmaceutical industry. To date, lots of methods have been explored to study incorporation of unnatural amino acids for widely applications such as protein-protein interaction, biophysical probes and creation of functionality [20]. Since Levine et al. [21] firstly introduced auxotrophic bacterial strains for incorporation of unnatural amino acids, this strategy has been studied extensively and become commercially available to numerous biology studies. In this strategy, the native translational apparatus recognized analogs of natural amino acids to provide an opportunity to accept unnatural amino acid analogues for incorporation to protein. Kiick et al. [22] successfully incorporated azido-homoalanine into protein (mDHFR) with high translational efficiency under help of Methionine-tRNA synthetases for acceptance of methionine analogs. To only incorporate azido-homoalanine into protein, natural methionine was removed from media to exclude side incorporation of methionine. This study developed here provided an opportunity to chemoselective modification and labeling of proteins in native conditions. In this study, we propose that the insertion of homoalanine into protein TM containing the EGF-like domains 456 (rTM₄₅₆) by auxotrophic E.Coli strain to attain an optimal platform for exploring site specification modification of recombinant TM (Figure 3.1).

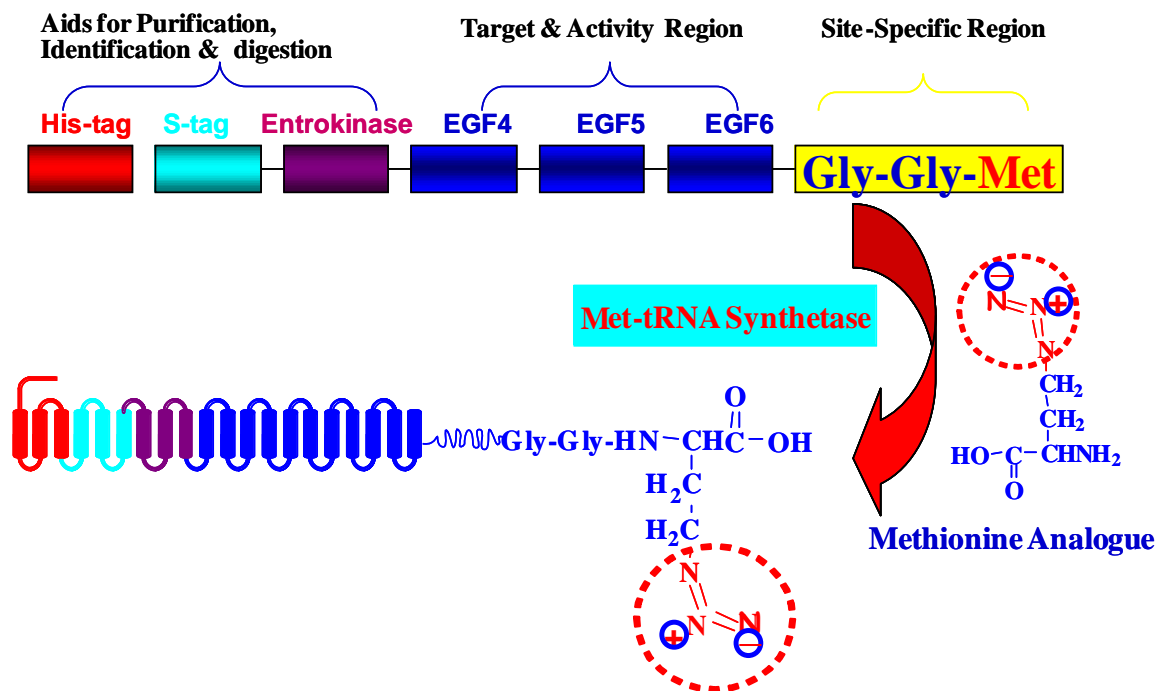


Figure 3.1 Schematic Structure of the Site-Specific Azide Functionalized Consecutive EGF-like Domains 4-6 of Human TM with His-Tag and S-Tag

3.2 Experimental

3.2.1 Materials and Methods

All solvents and reagents were from commercial sources and were used as received, unless otherwise noted. Deionized water was used as a solvent in all experiments. Human thrombomodulin cDNA clone was from ATCC (Manassas, VA), pET Dsb Fusion System 39b, competent cells, and kanamycin sulfate were from EMD Chemicals (Philadelphia, PA). Phusion® High-Fidelity PCR Kit, BamHI-HF, KpnI-HF, T4 DNA ligase and alkaline phosphatase were from New England Biolabs (Ipswich, MA). All plasmid purification kits were from QIAGEN Inc. (Chatsworth, CA). *E. coli*

strain B834 (DE3), plasmid pET-39b(+), and Site-Specific Enterokinase Cleavage and Capture kits were from Novagen (Madison, WI). The mouse monoclonal antibody specific to human TM was from COVANCE Corp. (Richmond, CA). Synthetic oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Purified recombinant human PC and human thrombin were from Haematologic Technologies Inc. (Essex Junction, VT). Human anti-thrombin, recombinant human TM and chromogenic substrate Spectrozyme PCa were from American Diagnostica Inc. (Stamford, CT). L-azidohomoalanine was from AnaSpec Inc. (Fremont, CA). Imidazole, sodium azide, other chemicals were from Sigma-Aldrich (USA). Fluorescence imaging of SDS-PAGE gels was performed using a Typhoon 9410 Variable Mode Imager (Amersham Biosciences, USA). Dialysis was performed using cellulose membranes with a molecular weight cutoff of 3.5 kDa with water as solvent. OD value was recorded on a Varian Bio50 UV-vis spectrometer.

3.2.2 Construction of Mutant of TM EGF-like (4-6) Domain

Plasmid Constructs. Recombinant thrombomodulin EGF₄₅₆ was prepared by expressing plasmid TM₄₅₆-N₃ in E. coli methionine auxotroph B834 (DE3). To encode thrombomodulin EGF 4-6 domains, forward primer: 5' CGC GGA TCC CGA CCC GTG CTT CAG A 3' and reverse primer: 5' GTT GCA AAA CAG CTG GCA CCT GTG 3' were used to create a mutant with C terminal methionine based on human thrombomodulin cDNA, in which unnatural amino acid could be incorporated into recombinant proteins to provide an azide group for chemoselective modification. To remove extra methionine in other sites of the mutant and to improve resistance to

oxidative inactivation and enzymatic activity, a point mutation was created to switch methionine to leucine at position 388 by using forward primer 5' CAC AGG TGC CAG CTG TTT TGC AAC 3' and reverse primer 5' CGC GGA TCC GAT TAC ATA CC C CCC AC 3'. PCR fragments containing the mutant sequences were ligated into pET 39 b(+) to make the expression plasmid TM₄₅₆GGM. The sequence of each mutant plasmid was confirmed by DNA sequencing.

3.2.3 Incorporation of Azides into TM₄₅₆ in E.coli and Protein Extraction

Media and materials for E. coli culture were prepared as following:

Table I. Media A for Single Colony Culture (10ml)

Materials	Volume	Sterilization
M9 medium (1 x)	10 mL	autoclave
MgSO ₄ (1 M)	10 µL	0.22 µm Filter
CaCl ₂ (1 M)	1 µL	0.22 µm Filter
20% D (+) Glucose	200 µL	0.22 µm Filter
Thiamine Hydrochloride (1 mg/mL)	10 µL	0.22 µm Filter
19AA W/O Methionine	400 µL	0.22 µm Filter
L-Methionine solution (20 mg/mL)	20 µL	0.22 µm Filter
Kanamycin (35 mg/mL)	10 µL	0.22 µm Filter

Table II. Media B for Scale Up Culture (1 L)

Materials	Volume	Sterilization
M9 medium (5 x) (autoclave)	200 mL	autoclave
dH ₂ O	720 mL	autoclave
MgSO ₄ (1 M)	1 mL	0.22 µm Filter
CaCl ₂ (1 M)	100 µL	0.22 µm Filter
20% D (+) Glucose	20 mL	0.22 µm Filter
Thiamine Hydrochloride (1 mg/mL)	1 mL	0.22 µm Filter
19AA W/O Methionine	40 mL	0.22 µm Filter
L-Methionine solution (20 mg/mL)	1 mL	0.22 µm Filter
Kanamycin (35 mg/mL)	1 mL	0.22 µm Filter

The recombinant TM₄₅₆-N₃ was expressed in E. coli as the following procedure:

1. Transform the constructed TM₄₅₆GGM to the appropriate E. coli strain and plate out on minimal medium plates. Incubate the plates overnight at 37°C.
2. Pick one colony and use it to inoculate 10 mL Media A. Incubate the culture on orbital incubator (180 rpm) overnight at 37°C.
3. Add the overnight culture to 1 L medium B plus 2mL L-Methionine (20 mg/mL) and incubate the culture at the appropriate temperature for induction until the absorbance at 600 nm is 0.8.
4. Centrifuge the cell suspension for 5 mins at 4,500 rpm at 4°C.
5. Washing cell pellets with 1 × M9 solution (autoclave)

6. Centrifuge for 5 mins at 4,500 rpm at 4 °C. Repeat 2 more times
7. Resuspend the pellet in 1 L Medium B (without methionine) and incubate for 1 hr at 37 °C to starve the cells
8. Add 2 mL Azidohomoalanine (20 mg/mL) and incubate for a further 30 mins.
9. Induce expression of the target protein by adding 1.2 mL IPTG (500 mM) to the medium and incubate the culture at 25 °C overnight.
10. Centrifuge the cell suspension for 10 mins at 8000 x g at 4 °C.
11. Store the cell pellet at -20 °C or -80 °C when not immediately used.

In the expression of rTM₄₅₆-N₃, a culture lacking methionine served as the negative control. After cells were harvested by centrifugation for 30 mins at 4 °C, lysis buffer (20 mM Tris-HCl, pH 8.0, and 300 mM NaCl) with the protease inhibitor PMSF (10 µg/mL) was added to a total volume of 50 mL. The cells were then sonicated every 2 min with a probe sonicator (Sonifier 450, Branson Ultrasonics, CT) on ice. Cell disruption was evidenced by partial clearing of the suspension. Then the broken cells were centrifuged 15000 x g for 30 mins and the supernatant was collected and directly applied to an immobilized metal affinity column (HisTrap FF) equilibrated with washing buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 20 mM imidazole). The column was washed with 10 column volumes of washing buffer, after which the column was flushed with 3 column volumes of cleavage buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 250 mM imidazole) to afford His-rTM₄₅₆-N₃. Finally, enterokinase was used for site-specific cleavage to remove the fusion tag and generate the target protein rTM₄₅₆-N₃.

A similar procedure was used for preparing positive control protein His-rTM and rTM₄₅₆ without incorporation of azide group. Briefly, the expression plasmid TM₄₅₆GGM was transformed into E. coli BL21 (DE3) cells. Bacteria were grown in LB media under the same conditions. rTM purification and fusion tag removal were performed under the same conditions.

3.2.4 Western Blot for Identification of rTM₄₅₆

After purification rTM₄₅₆ from HisTrap FF and followed by cleavage by enterokinase, SDS-PAGE gel electrophoresis was used to separate recombinant TM₄₅₆ with his tag and recombinant TM without his tag. 12 % Tris–glycine gels was made in the lab and used throughout. Electrophoresis was performed on XCell SureLock™ Mini-Cell Electrophoresis System (Invitrogen). The running buffer is 1X tris-glycine-SDS buffer. 20 µL solution was loaded into wells for each sample. Electrophoresis was run at 120 V for 2 hrs. After the gel has run, samples were transfer to nitrocellulose membrane at 90 V for 45 mins. The mouse monoclonal antibody specific to human TM was for binding of rTM. ECL kit (GE Healthcare) was use for detection of proteins.

3.2.5 Mass Spectrometry Analysis of Recombinant TM₄₅₆

After electrophoresis, the gels immediately was rinsed in dH₂O, and then immersed into Zinc-imidazole staining buffer (Bio-rad). After staining, the gel was rinsed completely in dH₂O. Then protein bands was cut from gel and destained according to standard protocols as instruction. Next, 2 or 3 pieces of gels were crushed and immersed

into 1mL extraction buffer (Formic acid/ water/2-propanol (1:3:2 v/v/v) (FWI) for 2hrs. Then samples were centrifuged at 15, 000 rpm for 20 mins. The supernatant was collected for MALDI-TOF analysis.

The extraction solution was lyophilized and a MALDI matrix solution (FWI saturated with 4-hydroxy-a-cyano-cinnamic acid (4HCCA)) was added to redissolve and prepare the protein for MS analysis using the dried-drop method of matrix crystallization [23]. The MALDI MS experiments were carried out on a Bruker Ultraflex III tandem time-of-flight (TOF/TOF) mass spectrometer (Bruker Daltonics, Billerica, MA), equipped with a Nd:YAG laser emitting at a wavelength of 355 nm. All spectra were measured in positive reflector mode. The instrument was calibrated externally with a poly(methyl methacrylate) standard prior to each measurement. Five different matrices were used: dithranol (DIT), 2,5-dihydroxybenzoic acid (DHB), and trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene] malononitrile (DCTB) dissolved in tetrahydrofuran (THF) at 20, 10, and 20 mg/mL, respectively, as well as 3-hydropicolinic acid (HPA) and R-cyano-4-hydroxycinnamic acid (CHCA) dissolved in acetonitrile(ACN)/water (v/v, 50/50) at 5 mg/mL. Samples were prepared by sandwich method involving depositing 0.5 μ L of matrix solution on the wells of a 384-well ground-steel plate, allowing the spots to dry, then depositing 0.5 μ L of each sample on top of a dry matrix spot, and adding another 0.5 μ L of matrix solution on top of the dry sample (sandwich method). MALDI experiments were performed using Bruker's LIFT mode. Data analysis was conducted with the flexAnalysis software.

3.2.6 Availability of Azide Moiety in the Recombinant TM₄₅₆

Samples of His-rTM₄₅₆-N₃ and rTM₄₅₆-N₃ were incubated with cyclooctyne functionalized Fluorescent dye Alexa Fluor® 647 DIBO (10 equiv, Invitrogen) in a phosphate buffer (pH 7.0) for 1 hr at room temperature. Then samples were dialyzed in Tris-HCl (20 mM, pH 7.4) for overnight. Next, samples were load into 12% tris-glycine gel for separation and carried out at 120 V for 1.5 hrs. Coomissie blue was used to stain gel. rTM₄₅₆ without azide was used as a negative control and subjected to the same reaction conditions. Finally, an imaging system (Typhoon 9410 Variable Mode Imager, Amersham Biosciences, USA) was used to analysize the fluorescent labeled proteins on the gel.

3.2.7 Cofactor Activity Assay of rTM

The activity of rTM₄₅₆ was defined as moles of produced APC per min by given amounts of rTM₄₅₆ protein in the presence of thrombin. All activations of protein C by rTM conjugates were performed for 60 min in 20 mM Tris-HCl buffer, pH 7.4, 100 mM NaCl, 0.1% BSA, and 5 mM Ca²⁺ at 37°C.

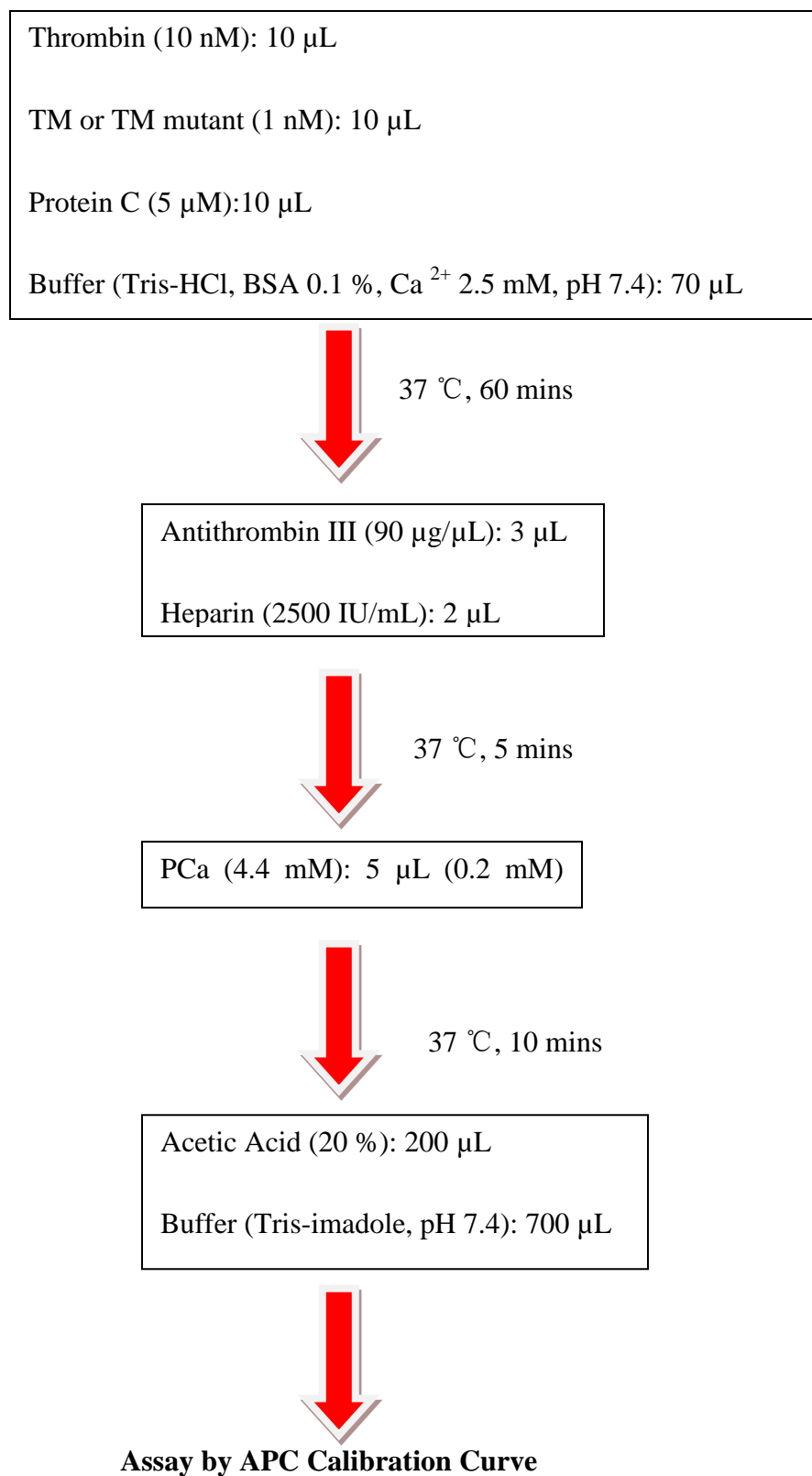


Figure 3.2 Flow Chart for Cofactor Activity Assay of TM

Typically, 20 nM thrombin and different rTM concentrations (1, 1.5, and 2 nM) were incubated in the assay buffer. After 60 mins, 3 μL antithrombin III (90 $\mu\text{g}/\mu\text{L}$) and 2 μL heparin (2500 IU/mL) were added into the solution to stop the reaction. The inhibition of protein C activation was completed within 5 mins at room temperature. The produced APC was measured through hydrolysis of a chromogenic substrate (Spectrozyme PCa) by comparing a standard curve (Figure 3.3), in which the concentration of APC to the rate of p-nitroanilide (pNA) formation was measured. The hydrolysis of Spectrozyme PCa was performed for 10 mins in the assay buffer at 37 °C, in which pNA was produced and the concentration measured by monitoring at $\lambda = 405 \text{ nm}$ with a spectrophotometer.

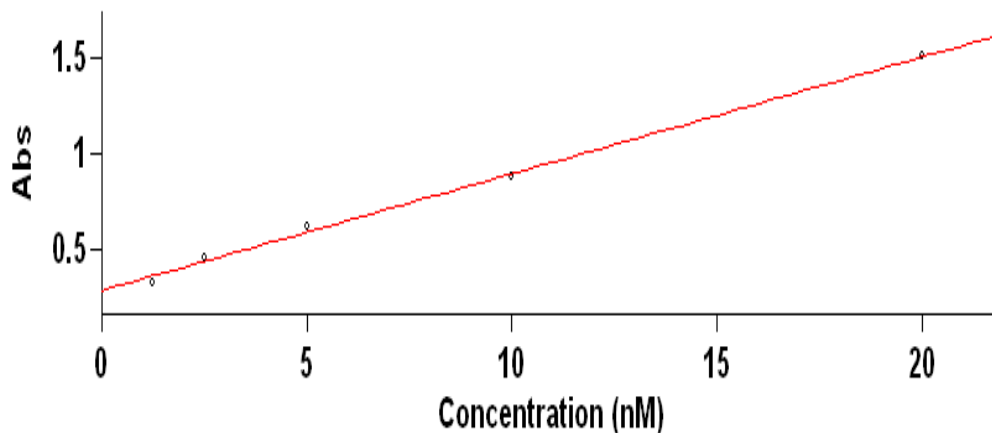


Figure 3.3 Standard Curve of Activated Protein C Assay

3.3 Results and Discussion

3.3.1 Construction of TM Mutant with Leucine Mutagenesis

A truncated TM fragment containing EGF4-6 with the insertion of a C-terminal non-natural methionine analogue was chosen as our target antithrombotic protein for site-specific conjugation [19]. Specifically, a truncated recombinant TM mutant containing amino acid sequences 349-492 with a Met-388-Leu substitution and a C-terminal linker GlyGlyMet-N₃ was constructed using site-directed mutagenesis (Figure 3.1).

In the construction of plasmid, two mutant fragments were created for mutagenesis from methionine to leucine by point mutation. This mutagenesis here could improve resistance to oxidation by using leucine instead of methionine at 388 [24]. The first fragment from Asp to inserted leucine has 118 base pairs; while the second fragments from leucine to methionine in the terminal has 323 base pairs. The sizes of those fragments have been confirmed by DNA analysis on agarose electrophoresis (Figure 3.4).

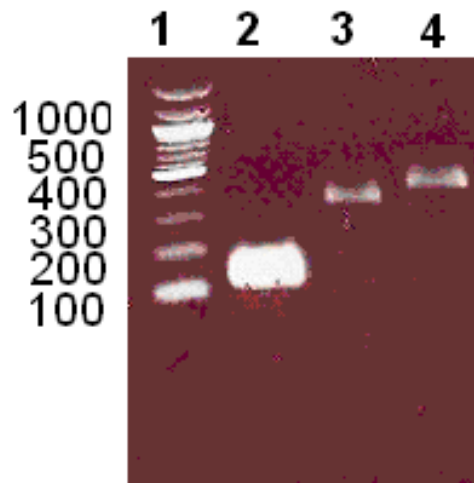


Figure 3.4 DNA Gel Analysis for Fragment: marker (lane 1), the fragment from Asp to inserted leucine (lane 2), from leucine to methionine (lane 3) and whole mutant (lane 4)

After construction of TM₄₅₆GGM, it was transformed into competent cells. Then, quantities of DNA encoding EGF-like 4-6 domain with a mutation of leucine at 388 have

been expressed in *E. coli* and harvested for DNA sequencing. The sequence of each mutated gene determined by DNA sequencing was following and was matched with its DNA sequence (marked by red and underline) as designed.

MKKIWLALAGLVLAFSASAAQYEDGKQYTTLEKPVAGAPQVLEFFSFFCPHCYQ
FEEVLHISDNVKKKLPEGVKMTKYHVNFMGGDLGKDLTQAWAVAMALGVEDK
VTVPLFEGVQKTQTIRSASDIRDVFINAGIKGEEYDAAWNSFVVKSLVAQQEKAA
ADVQLRGVPAMFVNGKYQLNPQGMDTSNMDVFVQQYADTVKYLSEKKGSTSG
SGHHHHHHSAGLVPRGSTAIGMKETAAAKFERQHMDSPDLGTDDDDKSPGFSST
MAIDPDPCFRANCEYQCQPLNOTSYLCVCAEGFAPIPHEPHRCQLFCNQTACPAD
CDPNTQASCECPEGYILDDGFICTDIDECENGGFCSGVCHNLPGTFCICGPDSAL
ARHIGTDCDSGKVDGGDSGSGEPPPSPTPGSTLTTPPAVGGM

3.3.2 Mass Spectrometry Characterization of rTM₄₅₆

In this study, recombinant TM₄₅₆ with His-tag and S-tag at the N-terminal and an azidohomolanine at the C-terminal was expressed in *E. coli*. Protein expression was confirmed by MALDI-TOF mass spectrometry (Figure 3.5). Other than Commisse blue, zinc –imidazole was used for protein staining. This staining method for protein detection is reversible and suitable for intact protein identification. Compared to other stains, it yields good efficiency and intact protein without need of digestion. The measured values fit for calculated molecular mass of His-rTM-N₃ (43192 D) and rTM-N₃ (16645 D).

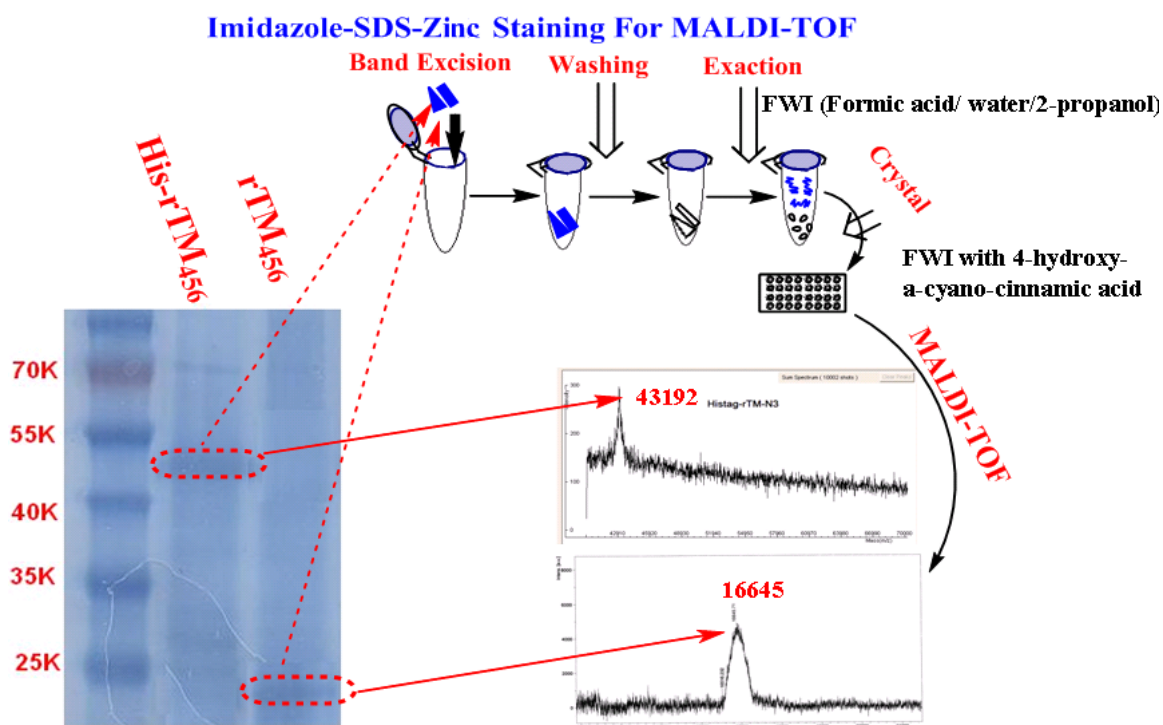


Figure 3.5 Mass Spectrometry for Characterization of His-rTM-N3 and rTM-N3

3.3.3 Availability of Azide Moiety in the Recombinant TM

The recombinants were further analyzed by SDS-PAGE and visualized by Coomassie blue staining (Figure 3.6A). The target recombinant TM fused to the leader sequence was clearly detected in the positive control cultures supplemented with azidohomoalanine, whereas it was not observed in the negative control culture. The recombinant TM with His-S tags was purified from the cell pellet by using metal-affinity chromatography with stepwise imidazole-gradient elution under native conditions. The rTM₄₅₆-N₃ was characterized by identification of a band at ~20 kDa on SDS-PAGE gels (Figure 3.6A), which was consistent with prior reports [19]. To investigate whether the azide moiety in the protein was reactive under copper-free click chemistry condition,

both His-rTM₄₅₆-N₃ and rTM₄₅₆-N₃ were incubated with cyclooctyne functionalized Fluorescent dye Alexa Fluor® 647 DIBO (10 equiv, Envirogen) in a phosphate buffer (pH 7.0) overnight at room temperature. rTM₄₅₆ without azide was used as a negative control and subjected to the same reaction conditions. Protein samples were dialyzed against the buffer to remove unreacted fluorescent dye and then analyzed by SDS-PAGE. The gel was stained with Coomassie blue (Figure 3.6A) and the fluorescent image from the gel were detected using an imaging system (Typhoon 9410 Variable Mode Imager, Amersham Biosciences, USA) (Figure 3.6B). As a result, the band for His-rTM₄₅₆-N₃ and rTM₄₅₆-N₃ were fluorescent, whereas no fluorescent image was detected from rTM₄₅₆ without an azide group. These results indicated that the azide moiety in the protein was reactive toward the cyclooctyne and could be conjugated to cyclooctyne-containing molecules under copper free click chemistry condition.

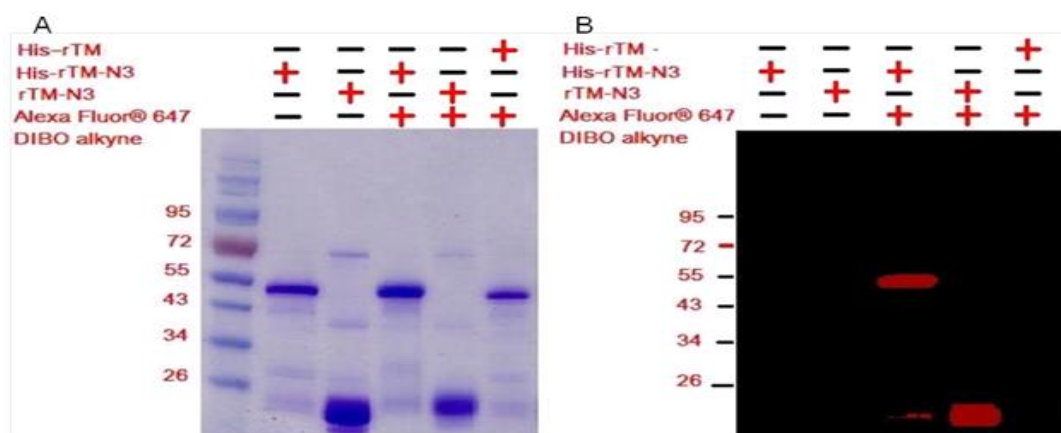


Figure 3.6 SDS-PAGE (12%) characterization of recombinant TM derivatives: A. Coomassie blue staining, B. Fluorescence scanning

3.3.4 Cofactor Activity Assay of rTM

In this study, protein C activation activities of the rTM were evaluated. The activity of rTM₄₅₆ was defined as moles of produced activated protein C per min by given amounts of rTM₄₅₆ and rTM₄₅₆ conjugates in the presence of thrombin. As shown in table III, recombinant His-TM₄₅₆-N₃ and TM₄₅₆-N₃ have shown similar protein c activity as commercial full TM. Taken together, rTM₄₅₆ has been successfully expressed in present study without apparent loss of protein C activity.

Table III. Protein C Activation Activity of rTM

	Full TM*	His-rTM-N ₃	rTM-N ₃
K _m (μ M)	0.60 \pm 0.15	0.80 \pm 0.2 (0.90 \pm 0.2)	0.90 \pm 0.2 (1.0 \pm 0.5)
k _{cat} (min ⁻¹)	0.20 \pm 0.03	0.28 \pm 0.05 (0.22 \pm 0.05)	0.24 \pm 0.04 (0.16 \pm 0.05)
k _{cat} /K _m (min ⁻¹ . μ M ⁻¹)	0.37 \pm 0.14	0.40 \pm 0.15 (0.26 \pm 0.1)	0.29 \pm 0.11 (0.16 \pm 0.05)

*commercial full TM of human, the data in parentheses from [19]

3.4 Conclusion

In this study, unnatural amino acid-azidohomoalanine was successfully incorporated into TM at the C terminal by directly acceptance of methionine analogs under help of Methionine-tRNA synthetases. This incorporation of azidohomoalanine provides an azide moiety for site specific conjugation through Staudinger ligation and Click chemistry, and makes an alternative to explore protein interaction and the effect of cell membrane to integral protein by membrane mimics.

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CHAPTER IV

SYNTHESIS AND CHARACTERIZATION OF ANTITHROMBOTIC LIPOSOMAL THROMBOMODULIN VIA STAUDINGER LIGATION

4.1 Introduction

Membrane proteins are becoming most investigated drug targets and have been studied extensively in biology sciences and pharmaceutical industry since they involve most of physiological process such as signal transduction, control of metabolic process and inspiration of immune systems. The tools today for studying and characterizing proteins have paved the way for membrane protein investigation and provide an opportunity to understand this kind of proteins and create novel drug targets. However, there are still some unexpected challenges to meet when membrane proteins are considered as novel drug targets, for example, extremely difficulty to purify, easily activation by removing from cell membrane and so on [1]. Liposome incorporated proteins provide protection from inactivation of membrane protein by mimicking real cell

membrane and become a very usefully tool to study physiological functions of membrane proteins [2, 3].

To date, liposome incorporated proteins are usually prepared by post-insertion method. In this strategy, target proteins are extracted from cell membrane and form micelles in buffer. Then micelles containing membrane proteins are incubated with performed liposome to form liposome incorporated membrane proteins by fusion interaction between hydrophobic tail of proteins and liposome. Detergents or organic solvents are required for their solubilization and purification [4]. However, detergents are difficult to remove completely, and can lead to toxicity *in vivo*. Furthermore, conformational change of proteins could happen and decrease activity or even loss of function due to random orientation [5]. Chemical modification methods, namely post-functionalization methods, provide an alternative to prepare liposome incorporated proteins, in which in most cases involve the coupling of biomolecules to the surface of preformed vesicles that carry functionalized lipid anchors [6]. Compared to post-insertion method, this strategy was designed to orient modification and reduced possibility of random confirmation. On the other hand, it provides an opportunity to incorporate proteins into liposome without help of hydrophobic tailor of membrane proteins, in turn, which makes it easier to prepare target proteins. So far, variable coupling methods have been studied for post-surface functionalization by using amide [7] or thiol-maleimide coupling [8], imine [9], hydrazone linkage [10] as well as bioorthogonal conjugation methods including native chemical ligation (NCL) [11], Staudinger ligation [12] or Click chemistry [13]. Among of current coupling methods, the Staudinger ligation is a widely used method for bioconjugation in lots of applications such as DNA labeling [14],

peptide-peptide conjugation [15], cell surface engineering [16] as well as drug delivery systems [12]. This reaction occurs in native condition with high yield and without any catalyst and is compatible with the unprotected functional groups of wide range of biomolecules.

Incorporation of non-natural amino acid into proteins provides a powerful tool to engineer proteins with additional functionalities for special applications, especially for those functionalities that cannot be introduced translationally into large proteins, as well as applications in protein tagging and protein conjugation. For instance, several kinds of methionine analogs containing azides into recombinant proteins for chemoselective modification have been incorporated into murine dihydrofolate reductase (mDHFR) [17]. This incorporation of UAAs containing azides provides very useful tool for a lots of application such as bioconjugation, vivo imaging, protein labeling or drug design and delivery by Staudinger ligation and Click chemistry. For example, site-specific PEGylation of human thrombomodulin has been performed to increase half-time in the blood via Staudinger ligation. [18]. An engineered LplA acceptor peptide (LAP) with an alkyl azide have been developed and further labeled in living mammalian cells which providing an opportunity to biochemical and imaging studies of cell surface proteins [19].

Particularly, TM is a type I membrane protein. The lipid bilayer in which it resides serves as an essential ‘cofactor’, locally concentrating and coordinating the appropriate alignment of reacting cofactors and substrates for protein C activation. Liposomes have been extensively studied as cell membrane model as well as carrier for delivering certain vaccines, enzymes, drugs, or genes to their active sites. Herein, we have explored regio- and chemoselective functionalization of recombinant

thrombomodulin (TM) to liposome surface via Staudinger ligation in order to mimic the native endothelial antithrombotic mechanism of both TM and lipid components and thus will provide a more forceful than current antithrombotic agent (Figure 4. 1).

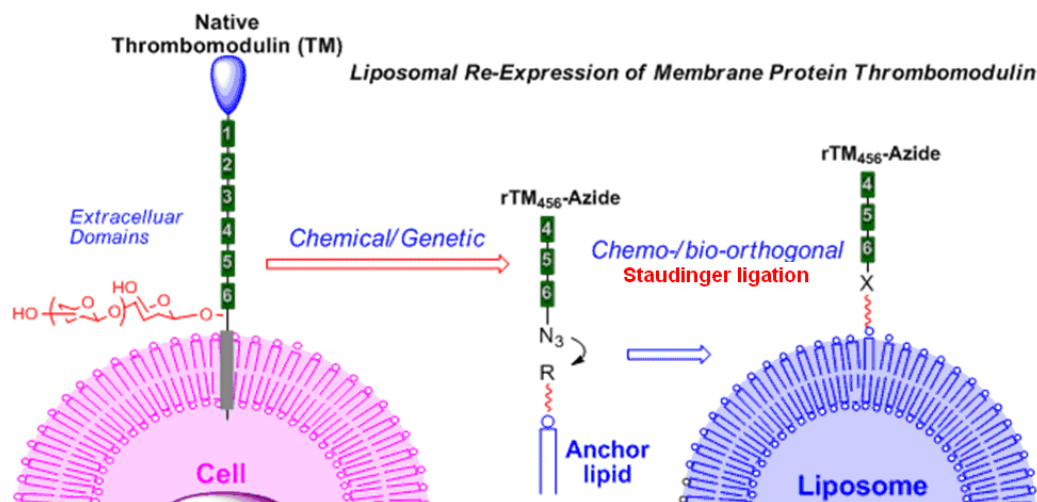


Figure 4.1 Schematic Illustration of Proposed Membrane Mimetic Re-expression of Membrane Protein TM onto Liposome

4.2 Experimental

4.2.1 Materials and Methods

All solvents and reagents were from commercial sources and were used as received, unless otherwise noted. Deionized water was used as a solvent in all experiments. The mouse monoclonal antibody specific to human TM was from COVANCE Corp. (Richmond, CA). Purified recombinant human PC and human thrombin were from Haematologic Technologies Inc. (Essex Junction, VT). Human anti-thrombin, recombinant human TM and chromogenic substrate Spectrozyme PCa were from American Diagnostica Inc. (Stamford, CT). L-azidohomoalanine was from AnaSpec Inc. (Fremont, CA). 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-

glycero-3-phosphoethanol-amine-N-[amino(polyethylene glycol)₃₄₀₀](ammonium salt) (DSPE-PEG₃₄₀₀-NH₂) were from Laysan Bio Inc. (Arab, AL). Cholesterol, dicyclohexylcarbodiimide (DCC), diphenylphosphino-4-methoxycarbonylbenzoic acid, hexaethylene glycol, toluene sulfonyl chloride, imidazole, sodium azide, N-hydroxysuccinimidobiotin, N,N-dimethylformamide and other chemicals were from Sigma-Aldrich (USA).

Mass spectrometry experiments were performed using QSTAR Elite mass spectrometer (Applied Biosystems, Foster City, CA). Data was conducted with Analyst® QS 2.0 software. Thin-layer chromatography (TLC) was performed on Whatman silica gel aluminum backed plates of 250 µm thicknesses on which spots were visualized with UV light or by charring the plate after dipping in 10% H₂SO₄ in methanol. Fluorescence imaging of glass slide was performed using a Typhoon 9410 Variable Mode Imager (Amersham Biosciences, USA). Dialysis was performed using cellulose membranes with a molecular weight cutoff of 3.5 kDa with water as solvent. ¹H NMR spectra were recorded at room temperature with a Varian INOVA 300 MHz spectrometer. In all cases, the sample concentration was 10 mg/mL, and the appropriate deuterated solvent was used as an internal standard. Dynamic Light Scattering was recorded with 90Plus particle size analyzer (BIC). IR spectra were measured on Bruker FT-IR spectrometer (Bruker Optics Inc, MA) using an attenuated reflectance attachment accessory. Fluorescent spectrum was measured with FluoroMax-2 (ISA)

4.2.2 rTM Expression and Purification from E. coli

rTM expression and purification were performed as the protocol in chapter IV. Similar procedure was used for preparation of positive control protein His-rTM and rTM without incorporation of azide group. Briefly, the expression plasmid TM₄₅₆GGM was transformed into E. coli BL21 (DE3) cells. For rTM with azide will be cultured and expressed in M9 medium plus azidohomoalanine. While control rTM without azide incorporation were grown in LB media and expressed in same conditions. rTM purification and fusion tag removal were performed at the same conditions

4.2.3 Synthesis of Anchor Lipid DSPE-PEG₃₄₀₀-Triphenylphosphine (DSPE-PEG₃₄₀₀-TP)

DSPE-PEG₃₄₀₀-NH₂ (100mg, 35.8 μ mol) was dissolved in 20 mL of CH₂Cl₂, and 0.2 mL of triethylamine was added. After stirring for 30 min at room temperature, a solution of succinimidyl 3-diphenylphosphino-4-methoxycarbonylbenzoate (22 mg, 47.6 μ mol) in 50 mL of CH₂Cl₂ was added. The reaction mixture was stirred at room temperature for 24 hrs and then concentrated under vacuum to give a residue, which was purified by silica gel chromatography with chloroform/methanol (4: 1, v/v) to afford product 1 (41 mg, 9.0 μ mol, 25 %). ¹H NMR (CDCl₃, 300 MHz) 8.06 (m, 1H), 7.79 (m, 1H), 7.44 (m, 1H), 7.66 (m, 2H), 7.52-7.42 (m, 2H), 7.28-7.34 (m, 8H), 6.64 (m, 1H), 5.19 (s, 1H), 4.34-4.20 (m, 3H), 3.95-3.80 (m, 3H), 3.80-3.50 (br. S, 44H, O-CH₂-CH₂-O), 3.40-3.20 (m, 3H), 2.28 (br.s, 4H), 1.52 (br.s, 4H), 1.36-1.20 (s, 32H), 0.89 (t, J, 6.9 , 6H), ³¹P NMR (CDCl₃, 121 MHz) : -2.7.

4.2.4 Preparation of Triphenylphosphine Functionalized Liposome

Triphenylphosphine functionalized liposome was prepared. DSPC and cholesterol at 2:1 mol ratio were used as the major components of all liposome. 1 mol % of anchor lipid DSPE-PEG₃₄₀₀-triphosphine was doped. In detail, the lipids mixture of cholesterol, mPEG₃₄₀₀, DSPE-PEG₃₄₀₀-TP (2: 1: 5%: 1% molar ratio) were first dissolved in chloroform. The solvent was gently removed on an evaporator under reduced pressure to form a thin lipid film on the flask wall and kept in a vacuum chamber overnight. Then, the lipid film was swelled in the dark with 2.5 mL PBS buffer (pH 7.4), followed 10 freeze-thaw cycles of quenching in liquid N₂ and then immersed in a 50 °C water-bath to form multilamellar vesicle suspension. Finally, the crude lipid suspension was extruded through polycarbonate membranes (pore size 600, 200, and 100 nm, gradually) at a 60 °C to afford small unilamellar vesicles.

4.2.5 Synthesis of rTM-Liposome Conjugates via Staudinger Ligation

rTM₄₅₆-N₃ (200 µg) was purified on a His-trap FF column and transferred into 50 mL of Tris-HCl buffer (Tris-HCl 20 mM, NaCl 150 mM, pH 7.4). The solution was added into 2 mL of triphenylphosphine-functionalized liposome (DSPE-PEG₃₄₀₀-TP, 1%) described above. The conjugation was then conducted at room temperature for up to 12 hrs with very gentle shaking under N₂ atmosphere. The unreacted rTM₄₅₆-N₃ was removed by gel filtration (1.5×20 cm column of Sephadex G-50) to generate rTM₄₅₆-liposome conjugate via Staudinger ligation DLS was used to monitor the integrity of the vesicles during and after the coupling reaction.

4.2.6 Protein Assay by Bradford Method.

1 mL purified rTM liposome conjugate from CL-6B column was collected in a acetone compatible tube, followed by adding 4 mL cool acetone (-20 °C). The mixture was vortexed and incubated for 60 mins at -20 °C, followed by centrifuging 10 mins at 13,000-15,000 × g. Then, supernatant was removed carefully and not dislodge the protein pellet. Next, the acetone was allowed to evaporate from the uncapped tube at room temperature for 30 mins. 1 mL Tris-HCl buffer (20 mM, pH 7.4) was added for the downstream process and vortexed thoroughly to dissolve protein pellet. Finally, the protein assay was performed as instruction manual of Bradford protein assay (Bio-Rad).

4.2.7 Stability Evaluation of Liposome during Staudinger Ligation and Liposomal rTM Conjugate

The stability of the liposomes during coupling reaction and the final liposomal-rTM₄₅₆ conjugate products were monitored by measuring fluorescent leakage in comparison to the same type of liposomes unmodified having encapsulated self-quenching concentrations of 5,6-carboxyfluorescein (85 mM) using FluoroMax-2 (ISA, NJ). Briefly, the lipid film composed of DSPC, cholesterol, mPEG₂₀₀₀, DSPE-PEG₃₄₀₀-TP (2: 1: 5%: 1% molar ratio) was swelled in the dark with 2.5 mL Tris-HCl buffer (Tris-HCl 20 mM, NaCl 150 mM, pH 7.4) containing 85 mM 5,6-Carboxyfluorescein (5,6-CF) to form the multilamellar vesicle suspension. The crude lipid suspension was extruded through polycarbonate membranes (Millipore sizes from 600 nm, 200 nm and 100 nm, successively) to produce small unilamellar vesicles with an average mean diameter of 120 ± 10 nm, as judged by DLS. Separation of the CF vesicles from non-entrapped CF was achieved by gel filtration chromatography, which involved passage through a 1.5×20

cm column of Sephadex G-50. Twenty μL of reaction solution was taken and mixed with 1980 μL of Tris-HCl buffer (pH 7.4), and then the fluorescent intensity was measured by using FluoroMax-2. A control experiment was conducted, in which recombinant TM with azide group was replaced with recombinant TM without azide group in equal amounts. To evaluate extent of fluorescence release upon disruption in the conjugation, 20 μL of 0.5 % Triton-100 solution was added into sample above to release all entrapped CF molecules from the liposomes and fluorescence intensity was measured.

5, 6-carboxyfluorescein (CF) released from liposomes in PBS (pH 7.4) buffer at room temperature was measured over time. The excitation and emission wavelengths of 5, 6-CF were 497 and 520 nm, respectively. The variation of the fluorescent intensity with release time was calculated according to the equation below

$$\text{Fraction of CF remaining in liposomes} = 1 - F/F_0$$

where F is the fluorescent intensity measured at any time during the experiment and F_0 is the total fluorescent intensity measured after disrupting liposomes completely with 0.5% Triton X-100 in PBS (pH 7.4) buffer.

4.2.8 Catalytic Cofactor Activity Assay of Liposomal rTM by Protein C Activity Assay

Catalytic cofactor activity assay of rTM derivatives including His-rTM₄₅₆-N₃, rTM₄₅₆-N₃, and liposome-rTM₄₅₆ Conjugates were analyzed by protein C assay. The activity of recombinant TM₄₅₆ was defined as moles of produced APC per min by given amounts of rTM₄₅₆ protein and rTM₄₅₆ conjugates in the presence of thrombin. All

activations of protein C by rTM conjugates were performed for 60 mins in 20 mM Tris-HCl buffer, pH 7.4, 100 mM NaCl, 0.1% BSA, and 5 mM Ca^{2+} at 37°C. Typically, 20 nM thrombin and different rTM concentrations (1, 1.5, and 2 nM) were incubated in the assay buffer. After 60 mins, 20 μL antithrombin III (3 $\mu\text{g}/\mu\text{L}$) and 20 μL heparin (1000 IU/mL) were added into the solution to stop the reaction. The inhibition of protein C activation was completed within 5 mins at room temperature. The produced APC was measured through hydrolysis of a chromogenic substrate (Spectrozyme PCa) by comparing a standard curve, in which the concentration of APC to the rate of p-nitroanilide (pNA) formation was measured. The hydrolysis of Spectrozyme PCa was performed for 10 mins in the assay buffer at 37 °C, in which pNA was produced and the concentration measured by monitoring at $\lambda = 405 \text{ nm}$ with a spectrophotometer.

4.3 Results and Discussion

4.3.1 Preparation of Triphenylphosphine Functionalized Liposome

The synthesis of triphenylphosphine functionalized liposomes starts with the synthesis of anchor lipid-DSPE-PEG₃₄₀₀-Triphenylphosphine. First, 3-diphenylphosphino-4-methoxycarbonylbenzoic acid NHS active ester was synthesized according the procedure [12]; then the NHS active ester was reacted with commercially available DSPE-PEG₃₄₀₀-NH₂ to form triphenylphosphine functionalized anchor lipid by amidation of primary amine in the lipid and NHS active ester (Figure 4.2). With the anchor lipid in hand, small unilamellar vesicles composed of DSPC and cholesterol (2:1 mol ratio), 5% mPEG₂₀₀₀, 1.0 mol % of the anchor lipid in Tris-HCl buffer (20 mM, pH 7.4) were prepared by rapid extrusion through polycarbonate membrane with pore size of

600, 200, and 100 nm diameter, sequentially at 65 °C. In the present study, mPEG₂₀₀₀ was used to improve stability of liposome in coupling reaction. This produced small unilamellar vesicles show an average mean diameter of 120±15 nm as determined by dynamic light scattering.

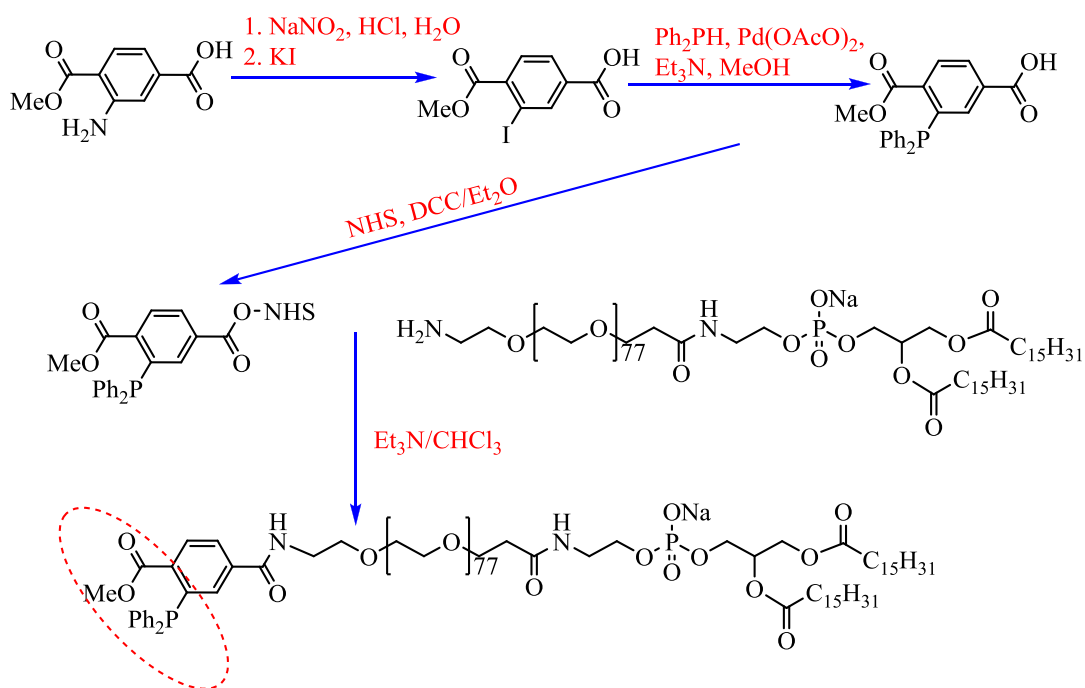


Figure 4.2 Scheme for Synthesis of DSPE-PEG3400-Triphenylphosphine

4.3.2 Synthesis of rTM₄₅₆-Liposome Conjugates via Staudinger Ligation

In order to make a membrane mimetic TM conjugate, we then investigated the selective Staudinger ligation for conjugation of the rTM₄₅₆-N₃ onto triphenylphosphine functionalized liposomes. Briefly, liposome incorporation of anchor lipid for Staudinger ligation was prepared. Then, rTM₄₅₆ was incubated with liposome for 12 hrs and followed by purification through CL-6B column chromatography to provide liposomal rTM₄₅₆ conjugate. As In the present study, the chemically selective post-modification

approach was investigated to generate the proposed rTM-liposome conjugate by taking the advantage of the efficient utilization of rTM on the outer leaflet and avoiding solubility problems in the formation of a lipid-protein conjugate. Furthermore, compared to traditional direct liposome formulation for reagent delivery, the strategy used here provides an opportunity to reduce inevitable loss of targets ligands when they are facing the enclosed aqueous compartment and thus become unavailable for intended interaction. Control experiment between rTM₄₅₆ and liposome without anchor lipid was conducted with the same procedure. As shown in figure 4.3, the reaction between rTM₄₅₆ and liposome with anchor lipid formed was observed as a new band after conjugation (Figure 4.3A), while control experiment between rTM₄₅₆ and liposome without anchor lipid did not exhibit any new bands (Figure 4.3B). These results indicated successful liposome modification with rTM-N₃ via Staudinger ligation.



Figure 4.3 Western Blot for Monitoring Conjugation by Staudinger Ligation. A: rTM-N₃ with liposome including anchor lipid; B: rTM-N₃ with liposome without anchor lipid

The conjugation efficiency of the click conjugation was evaluated by a given amount of rTM₄₅₆-N₃ and fixed anchor lipid (DSPE-PEG₃₄₀₀-TP) concentration in the liposome (1% of the lipids in liposome), in which DSPE-PEG₃₄₀₀-TP was in excess to rTM₄₅₆-N₃. The grafted amount of rTM₄₅₆-N₃ onto the liposome was calculated after gel filtration purification (CL 6B, GE Healthcare) by detecting unreacted rTM₄₅₆-N₃ in the

buffer and rTM₄₅₆-PEG₃₄₀₀-DSPE formed, respectively. Acetone was used to precipitate proteins allowing for the physical separation of protein pellet from the liposomal solution. The pellet was then redissolved in Tris-HCl buffer (pH 8.0) and detected by the Bradford protein assay (Bio-Rad). As a result, the conjugation yield was 21% by detecting the rTM-liposome conjugate formed and 30% by detecting the unreacted rTM-N₃ left, respectively. The gap between the calculated values of direct and indirect methods might come from loss of unreacted rTM₄₅₆-N₃ during the process of purification for the indirect.

4.3.3 Stability Evaluation of Liposome during Staudinger Ligation and Liposomal rTM Conjugate

Dynamic light scattering (DLS) was used to monitor the reaction and verify the integrity of the liposome during and after the Staudinger ligation reaction. As shown in Figure 4.4, there was no significant change in the size of the vesicles during and after conjugation reaction. The average of liposome size increased from 112 ± 10 nm (Figure 4.4A) to 119 ± 15 nm (Figure 4.4B). After separation of the rTM-liposome conjugate from the reaction solution, the rTM-liposome conjugate did not show size change (Figure 4.4C). Furthermore, there was no size change of control experiment in the presence of rTM₄₅₆ without azide group (Data not shown here). This result indicated that the liposomes were intact during the reaction and purification processes.

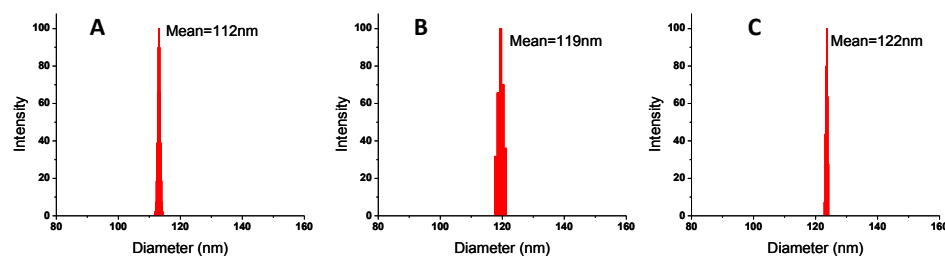


Figure 4.4 DLS Evaluation of Liposome Stability during Staudinger Ligation Conjugation between Liposome with rTM456-N3: A. before conjugation; B. after conjugation; C. purified conjugate

Fluorescent dye leakage of the same type of liposomes was conducted to further test whether the conjugation could provoke leakage in the liposomes during the reaction and the stability of liposomal conjugate. In present study, self-quenching concentrations of 5,6-carboxyfluorescein (5,6-CF, 85 mM) was encapsulated into inner of liposome in the preparation of liposome, then followed by passing through G-50 to remove free fluorescent dye. The leakage of fluorescent day was monitored using FluoroMax-2 (ISA) spectrometer. Based on the fluorescence quenching determinations in coupling reaction (Figure 4.5A) and rTM-liposome conjugate releasing assay (Figure 4.5B), we demonstrated that no apparent leakage was triggered by the conjugation reaction compared to the liposomes incubated in the presence of rTM₄₅₆ without azide group (Data not shown here). This fluorescence intensity unchanging indicated that no liposome disruption occurred during the conjugation even extension 24 hrs. In addition, the conjugation of the rTM to the liposome surface may have enhanced the liposome stability as it exhibited significantly slower dye release between 14 days to 25 days as compared to the control liposome treated with rTM without azide (Figure 4.5B). These results indicated the compatibility of the Staudinger ligation for rTM-liposome formation, and

the rTM-liposome conjugate was quite stable and could be used for further activity evaluation.

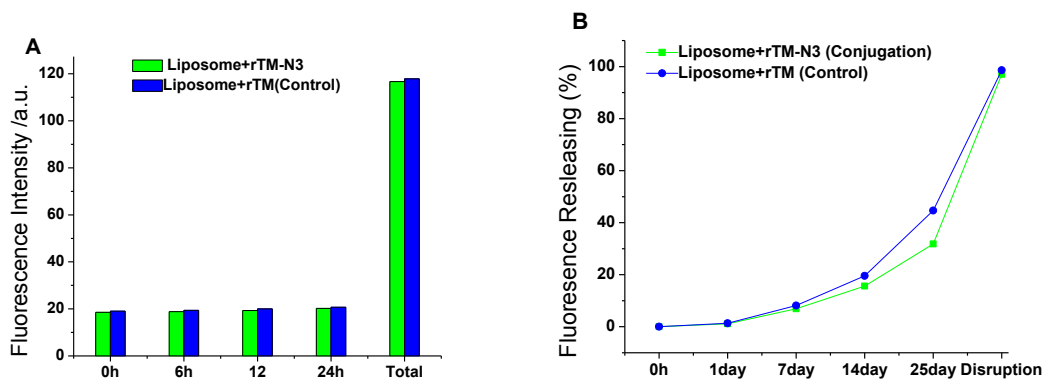


Figure 4.5 Evaluation of Stability of Liposome during Staudinger Ligation Reaction (A) and Stability of rTM-Liposome Conjugate (B) by Monitoring Fluorescent Dye 5,6-CF Releasing from Liposomes. Disruption of liposomes (total in A and disruption in B) was conducted by adding Tris-HCl buffer containing 0.5% Triton X-100 surfactant into the liposome solutions.

4.3.4 Catalytic Activity Assay of rTM Conjugates

In this study, protein C activation activities of the rTM conjugates were evaluated. The activity of rTM₄₅₆ was defined as moles of produced activated protein C per min by given amounts of rTM₄₅₆ and rTM₄₅₆ conjugates in the presence of thrombin. All protein C activation assays by rTM₄₅₆ and rTM₄₅₆ conjugates were performed for 60 mins in a buffer with 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% BSA, and 5 mM Ca²⁺ at 37 °C, as previously reported [20]. The catalytic activity of rTM₄₅₆ and rTM₄₅₆ conjugates were evaluated via the activation of varying human protein C concentration by rTM₄₅₆ and rTM₄₅₆ conjugates in the presence of thrombin and calcium. The rate of protein C

activation was linear with time until at least ~20% of the protein C was activated. Reaction conditions (TM concentration or incubation time) were adjusted so that less than 10 % of the protein C was activated to ensure that the amount of activated protein C by TM was in the linear range. TM-phospholipid interactions have been widely investigated before by different research group. The fact has been proven that phospholipids play a positive effect on the enhancement of activation of protein C by the thrombin-thrombomodulin complex in the surface of islet [21] or lipid vesicles [20, 22] by physical incorporation. The activation of protein C by the thrombin-thrombomodulin complex has comparable second-order rate constants by determination of the kinetic parameters of activation of protein [23]. Therefore, it is the k_{cat}/K_M value that allows direct comparison of the effectiveness of different forms of TM in different tissues or organisms toward protein C. Typically, catalytic activity of TM will change by 1) altering the k_{cat} , 2) altering K_M for protein C. As shown in the Table 4.1, recombinant His-TM₄₅₆-N₃ and TM₄₅₆-N₃ do not have apparent loss of activity while after conjugation, the value of k_{cat}/K_M of rTM-liposome conjugate has a twice to which of His-TM₄₅₆-N₃ or rTM₄₅₆-N₃ although there was no apparent change of k_{cat} , this change of k_{cat}/K_M mainly resulted from decreasing of K_M which is responsible for affinity of rTM for protein C. This result was consistent with a previous report in which the enhancement of k_{cat}/K_M was from increase of affinity of rTM for protein C [20]. This increasing of affinity may be the result of using liposome as a platform which has a beneficiary effect on the configuration of the conjugated rTM for enhancing thrombin and protein C binding.

Table IV. Protein C Activation Activity of rTM and its Conjugate via Staudinger ligation

	Full TM*	His-rTM-N ₃	rTM-N3	rTM-liposome
K _M (μM)	0.60±0.15	0.80±0.2 (0.90±0.2)	0.90±0.2 (1.0±0.5)	0.31±0.15
k _{cat} (min ⁻¹)	0.20±0.03	0.28±0.05 (0.22±0.05)	0.24±0.04 (0.16±0.05)	0.24±0.07
k _{cat} /K _M (min ⁻¹ .μM ⁻¹)	0.37±0.14	0.40±0.15 (0.26±0.1)	0.29±0.11 (0.16±0.05)	0.77±0.26

*commercial full TM of human, the data in parentheses are from Chaikof et al [18].

4.4 Conclusion

We demonstrated here the first example of biomimetic thrombomodulin conjugates which were synthesized site-specifically at the C-terminal with a chemical tag via Staudinger ligation. This liposomal rTM conjugate exhibited similar catalytic activity of natural TM with a potential to develop a more forceful antithrombotic agent. Furthermore, the proposed membrane-mimetic re-expression of recombinant TM onto liposome will provide a rational design strategy for facilitating studies of membrane protein functions and generating a protein-based drug

4.5 References

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CHAPTER V

SYNTHETIC BIO-INSPIRED THROMBOMODULIN CONJUGATES VIA COPPER-FREE CLICK CHEMISTRY FOR EXPLORING MEMBRANE PROTEIN'S SPECIFIC ACTIVITY

5.1 Introduction

TM is a glycoprotein that contains chondroitin sulfate glycosaminoglycans (CSGAG) attached to the D3 domain, which contributes to TM's thrombin binding, stability, and plasminogen activation activities [1]. Synthetic glycopolymers with multiple copies of sugar moieties have shown very promising results when used as natural oligosaccharide mimics [2]. Glycoengineering aimed at adding carbohydrates to proteins to alter pharmacokinetic properties, such as increasing in vivo activity and prolonging the duration of action, has become a developing field in regards to the enhancement of protein therapeutics [3, 4]. Recently, covalent attachments of synthetic glycopolymers having multiple copies of sugar moieties have been explored for therapeutic applications [5, 6]. Therefore, we hypothesized that modification of

recombinant TM containing the EGF-like domains 456 (rTMEGF₄₅₆) with glycopolymers may mimic the natural glycosylation on the TM (Figure 5.1a) and that such modifications may improve recombinant TM's activity in vivo, such as reducing immunogenicity and extending plasma half-lives.

Since TM is a membrane protein, it would be logical to develop a membrane mimetic conjugate of TM to mimic the native endothelial antithrombotic mechanism associated with cell membrane lipid components, thereby creating a more efficacious agent than current soluble TM without the membrane domain [7]. In addition, liposomes have been extensively studied as cell membrane models and as carriers for delivering vaccines, enzymes, drugs, and genes to sites of action [8]. In this study, we envisioned that rTM-liposome conjugates containing rTMEGF₄₅₆ may provide a rational design strategy for facilitating studies of membrane TM's functions while generating an optimal platform for exploring membrane protein-based drugs (Figure 5.1b).

In the past, the production of such protein-conjugates, to serve as effective biotherapeutics, was hindered by nonspecific conjugation reactions, resulting in a variety of isoforms, which potentially eliminate the protein's proper activity for the intended use. However, more advanced protein engineering and new bioconjugation techniques now permit to include unique attachment sites within peptides for numerous applications in protein engineering and functional studies. For example, the introduction of chemically unique groups into proteins by means of non-natural amino acids allows for site-specific functionalizations [9]. Previously, an azido-containing rTM construct was reported for the site-specific conjugation with a methoxy-terminated polyethylene glycol (mPEG) derivative via Staudinger ligation [10]. Site-specific immobilization of a rTM derivative

at the C-terminus through click chemistry with a suitably engineered alkyne PEG glass slide was also demonstrated [7]. The Cu(I)-catalyzed click chemistry has become of great use in modifying proteins and other macromolecules [6, 11]. However, a disadvantage of this reaction is the potential presence of residual copper, which can be potentially toxic, in the product intended for biological application [12]. Recently, to avoid the use of the potentially toxic copper catalyst, copper-free click chemistry has emerged as a popular bioorthogonal ligation strategy [13]. This reaction has been employed to solve many problems in chemical biology, pharmaceutical science and materials science. It has been used as a versatile chemistry for biomolecule modification [14], cell surface modification [15] and biomaterial fabrication [16] applications. In this study, we have explored regio- and chemoselective glyco- and liposomal functionalization of a recombinant TM456 derivative at the C-terminus through copper-free click chemistry in order to afford biomimetic TM conjugates, which are expected to be potential anticoagulants with enhanced pharmacokinetic properties (Figure 5.1).

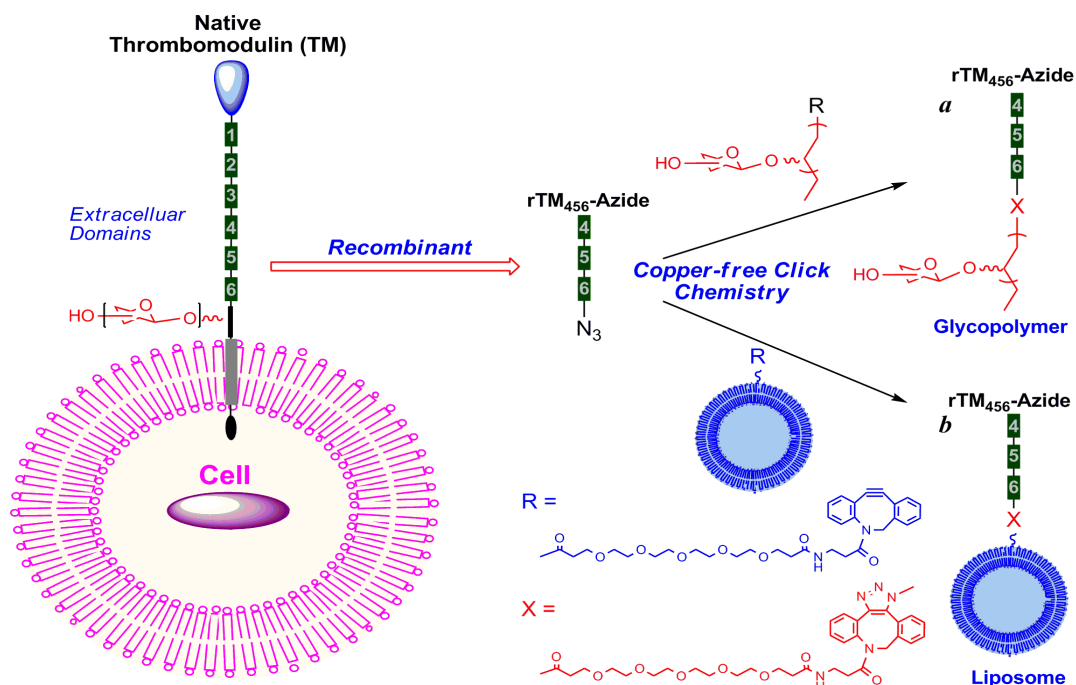


Figure 5.1 Schematic Illustration of Syntheses of Biomimetic TM Conjugates: A. rTM-glycopolymer conjugate; B. rTM-liposome conjugate via copper-free click chemistry modifications. rTM₄₅₆: recombinant TM containing EGF-like domains 4, 5, and 6.

5. 2 Experimental

5.2.1 Materials and Methods

All solvents and reagents were from commercial sources and were used as received, unless otherwise noted. Deionized water was used as a solvent in all experiments. The mouse monoclonal antibody specific to human TM was from COVANCE Corp. (Richmond, CA). Purified recombinant human PC and human thrombin were from Haematologic Technologies Inc. (Essex Junction, VT). Human anti-thrombin, recombinant human TM and chromogenic substrate Spectrozyme PCa were from American Diagnostica Inc. (Stamford, CT). L-azidohomoalanine was from AnaSpec

Inc. (Fremont, CA). 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanol-amine-N-[amino(polyethylene glycol)₂₀₀₀](ammonium salt) (DSPE-PEG₂₀₀₀-NH₂) were from Avanti Polar Lipids (Alabaster, AL). DBCO-PEG₄-NH₂ was from Click Chemistry Tools (Scottsdale, AZ). Glyco-Staining kits were from Geno Technology (St Louis, MO). Cholesterol, dicyclohexylcarbodiimide (DCC), diphenylphosphino-4-methoxycarbonylbenzoic acid, hexaethylene glycol, toluene sulfonyl chloride, imidazole, sodium azide, N-hydroxysuccinimidobiotin, N,N-dimethylformamide and other chemicals were from Sigma-Aldrich (USA).

Mass spectrometry experiments were performed using QSTAR Elite mass spectrometer (Applied Biosystems, Foster City, CA). Data was conducted with Analyst® QS 2.0 software. Thin-layer chromatography (TLC) was performed on Whatman silica gel aluminum backed plates of 250 µm thicknesses on which spots were visualized with UV light or by charring the plate after dipping in 10% H₂SO₄ in methanol. Fluorescence imaging of SDS-PAGE gels was performed using a Typhoon 9410 Variable Mode Imager (Amersham Biosciences, USA). Dialysis was performed using cellulose membranes with a molecular weight cutoff of 3.5 kDa with water as solvent. ¹H NMR and ¹³C-NMR spectra were recorded at room temperature with a Varian INOVA 300 MHz spectrometer. In all cases, the sample concentration was 10 mg/mL, and the appropriate deuterated solvent was used as an internal standard. Dynamic Light Scattering was recorded with 90Plus particle size analyzer (BIC). IR spectra were measured on Bruker FT-IR spectrometer (Bruker Optics Inc, MA) using an attenuated reflectance attachment accessory. Fluorescent spectrum was measured with FluoroMax-2.

5.2.2 rTM Expression in and Purification from *E. coli*.

rTM expression and purification were performed as the protocol in chapter IV. Similar procedure was used for preparation of positive control protein His-rTM and rTM without incorporation of azide group.

5.2.3 Synthesis of p-Amido[tetra(ethylene glycol)]-N-Dibenzylcyclooctynephenyl- β -D-Galactopyranoside (DBCO-PEG₄-CONH-Ph-Gal)

To a vial containing 3.2 mg of p-aminophenyl- β -D-galactopyranoside and a stir bar, 0.1 mL Et₃N was added and the reaction mixture was then allowed to stir for 15 min at room temperature. After 15 min, then a 1 mL solution of dry DMF containing 9 mg (1.1 eqv) DBCO-PEG₄-NHS ester was added via syringe. The vial was then flushed with N₂ (g) and capped to stir at room temperature for 24 h. The reaction prior to work up was checked by TLC 1:4 MeOH/CHCl₃ against the starting materials to confirm reaction completion. Then the solvent in the vial was reduced to near dryness under vacuum. The residue was then chromatographed on silica gel using 1:4 MeOH/Chloroform solution as eluent. The isolated compound, exhibiting long-wave UV-Vis fluorescence (R_f ~ 0.45, 1:4 MeOH/Chloroform), was collected and reduced to dryness achieving DBCO-PEG₄-CONH-phenylgalactose (4.7 mg, 47%). ¹H NMR (CDCl₃, 300 MHz) δ : 7.64 (d, 1H, H-Ar of DBCO), 7.46 – 7.34 (m, 12H, H-Ar of DBCO and Ph), 7.24 (d, 1H, H-Ar of DBCO), 7.06 (d, 2H, H-Ph), 5.11 (d, 1H, H1-Gal), 4.84 (d, 2H, CH₂ of DBCO), 3.92 (s, 1H, H-Gal), 3.80-3.47 (m, 5H, H-Gal), 3.55 (s, 16H, CH₂-PEG), 3.23 (m, 1H), 3.09 (m, 1H), 2.92 (m, 1H), 2.60 (t, 4H, CH₂CO₂), 2.42 (m, 1H), 2.27 (m, 2H), 2.02 (m, 1H).

FTIR (cm⁻¹): 3341, 3066 (C≡C), 2875, 2486 (C≡C), 1715, 1646, 1508, 1456, 1397, 1226, 1076, 832.

5.2.4 Synthesis of DBCO-Functionalized Glycopolymer based on Cyanoxyl-Mediated Free Radical Polymerization

First, lactose-based O-cyanate chain-end functionalized glycopolymer was synthesized from a lactose acrylamide derivative via cyanoxyl mediated free radical polymerization. The procedure is described in detail in previously reported work [17]. Briefly, Sodium nitrite (7 mg, 1.0 mmol) and 4-chloroaniline (11 mg, 0.09 mmol) was dissolved in 3ml mixture of H₂O/THF (1:1, V/V), followed by adding HBF₄ (122 mg, 1.5 mmol) to incubate for 30 mins. Next, acrylated lactose monomer (90 mg, 13 μmol) NaOCN (27 mg, 0.42 mmol) and acryamide (105 mg, 13 μmol) was added sequently and incubated over 16 h at 60 °C, followed by dialysis (3500 Da, MW cutoff) to afford lactose based O-Cyanate Chain-end glycopolymer (275 mg, 65 %).

Then, DBCO Functionalized glycopolymer was synthesized directly from the Lactose-based O-cyanate chain-end functionalized glycopolymer by dissolving 50 mg in 0.1 M sodium bicarbonate buffer (pH 8.3), and then by adding DBCO-PEG₄-Amine (5 mg, 9.5 μmol) dissolved in 200 μL DMSO slowly. The mixture was allowed to stir for 12 h at room temperature, followed by dialysis (3500 Da, MW Cutoff) to remove any unreacted DBCO-PEG₄-Amine providing the DBCO functionalized glycopolymer (35 mg, 70%).

5.2.5 Synthesis of 1,2-Distearoyl-sn-glycero-3-phosphoethanol amine-N-[(Polyethylene glycol)₂₀₀₀]-N-[Tetra(ethylene glycol)]-N-Dibenzylcyclooctyne (DSPE-PEG₂₀₀₀-DBCO)

To a dry 25 mL round bottom flask containing a stir bar, DSPE-PEG₂₀₀₀-NH₂ (94.4 mg, 0.034 mmol) was added and the flask was then equipped with a 3-way stopcock and placed under vacuum for 20 min. The sealed flask, equipped with a balloon, was backfilled with Ar(g) and then 3 mL of dry chloroform was added via syringe to dissolve the contents under stirring. After the solid was dissolved, while stirring, DBCO-PEG₄-NHS ester dissolved in 1 mL of dry chloroform (20 mg, 0.029 mmols) was added via syringe slowly, dropwise over 5 min, and the mixture was allowed to stir under Ar(g) at room temperature for 15 min. Then, via syringe, triethylamine (0.05 mL, 0.36 mmols) was added and the reaction was monitored via TLC (1:10 MeOH: CHCl₃) over a 48 h period. After 48 h, or upon reaction completion, the reaction mixture was then concentrated under reduced pressure and purified via silica gel column chromatography using methanol/chloroform (1:10) as eluting solvent affording pure DSPE-PEG₂₀₀₀-DBCO (0.077g, 79% yield). ¹H NMR (CDCl₃, 300 MHz) δ: 8.06 (s, 3H), 7.68 – 6.6 (d, 8H), 5.21 (s, 1H), 5.13 (d, 2H), 4.35 (d, 2H), 4.21 (t, 2H), 3.99 (m, 6H), 3.73 – 3.55 (m, 204H), 3.10 (t, 1H), 2.70 (t, 2H), 2.47 (t, 2H), 2.29 (t, 4H), 1.59 (t, 4H), 1.35 (t, 4H), 1.26 (t, 52H), 0.88 (t, 6H). ¹³C NMR (CDCl₃, 75 MHz) δ: 173.4, 173.0, 136.1, 132.1, 129.1, 128.6, 127.8, 125.6, 108.0, 93.2 (alkyne), 70.6 (PEG), 67.2, 55.5, 45.7, 39.1, 36.8, 34.1, 32.0, 29.71, 24.9, 22.7. FTIR (cm⁻¹): 3490, 2915, 2870, 2850, 2091, 1714, 1651, 1542, 1466, 1349, 1249, 1092, 948, 843, 720.

5.2.6 Site-Specific Galactose-Modification of His-rTM₄₅₆-N₃ via Copper-Free Click Chemistry

To 100 μ L of 20 mM Tris-HCl buffer (pH 7.4) with 10 μ g of recombinant His-TM₄₅₆-N₃, DBCO-PEG₄-CONH-Phenyl-galactose (1 mg) in 20 μ L DMSO was added, and the mixture was stirred for 12 hrs at room temperature. SDS-PAGE gel (12%) was used to separate the reaction mixture, followed by glyco-staining and Coomassie blue staining to confirm reaction completion. Staining procedures were followed according to the manufacturers' instructions.

5.2.7 Site-Specific Glycopolymer-Modification of His-rTM₄₅₆-N₃ via Copper-Free Click Chemistry

To 100 μ L of 20 mM Tris-HCl buffer (pH 7.4) with 10 μ g recombinant His-TM₄₅₆-N₃, DBCO-PEG₄-Glycopolymer (2 mg) in 20 μ L DMSO was added, and the mixture was stirred for 12 h at room temperature. SDS-PAGE gel (12%) was used to separate the reaction mixture, followed by glyco-staining and Coomassie blue staining to confirm this reaction. Staining procedures were followed according to the manufacturers' instructions.

5.2.8 Conjugation of His-rTM-N₃ to Anchor Lipid (DSPE-PEG₂₀₀₀-DBCO) via Copper-Free Click Chemistry

To a solution of 50 μ g recombinant TM₄₅₆ in 400 μ L 20mM Tris-HCl buffer (pH 7.4), 0.5 mg DSPE-PEG₂₀₀₀-DBCO in 20 μ L DMSO was added, and then the mixture

was stirred for 12hrs at room temperature. The efficiency of click conjugation between anchor lipid and recombinant TM₄₅₆ has been evaluated given amount of rTM₄₅₆ and anchor lipid over time. This conjugation was monitored by 12% SDS-PAGE gel and visualization of protein bands by Coomassie blue staining. At the same time, western blot was used to confirm the conjugate. Control experiment was conducted by incubation of same amount of anchor lipid into rTM₄₅₆ without azide group solution of Tris-HCl buffer.

5.2.9 Preparation of DBCO-Functionalized Liposomes

DSPC (15 mg, 20.43 μ mol), cholesterol (4 mg, 10.2 μ mol), mPEG₂₀₀₀-DSPE (4.6 mg, 1.67 μ mol), DBCO-PEG₂₀₀₀-DSPE (2.2 mg, 0.65 mol) (2:1:5%:2% molar ratio) were dissolved in 3.0 mL chloroform. The lipid mixture was dried onto the wall of a 100 mL round-bottom flask by removing the solvent gently using a rotate evaporator under reduced pressure followed by placing the vessel under vacuum overnight to form a thin lipid film on the flask wall. Then the lipid film was swelled in the dark with 2.5 mL Tris-HCl buffer (Tris-HCl 20 mM, NaCl 150 mM, pH 7.4) to form a multilamellar vesicle suspension. Ten freeze-thaw cycles using liquid N₂ followed by immersion in a 65 °C water bath were performed. The crude lipid suspension was extruded through polycarbonate membranes (Millipore size from 600 nm, 200 nm and 100 nm, successively) to produce small unilamellar vesicles with an average mean diameter of 120 ± 10 nm, as determined by DLS.

5.2.10 Synthesis of rTM-Liposome Conjugates via Copper-free Click Chemistry

His-rTM₄₅₆-N₃ (200 µg) was purified on a His-trap FF column and transferred into 50 mL of Tris-HCl buffer (Tris-HCl 20 mM, NaCl 150 mM, pH 7.4). The solution was added into 2 mL of DBCO-functionalized liposome (DBCO-PEG₂₀₀₀-DSPE, 2%) described above. The click conjugation was then conducted at room temperature for up to 9 h with very gentle shaking. The unreacted His-rTM₄₅₆-N₃ was removed by gel filtration (1.5×20 cm column of Sephadex G-50) to generate His-rTM₄₅₆-liposome conjugate via copper-free click chemistry. As in the same procedures above, the targeted rTM₄₅₆-liposome conjugate was synthesized by releasing the His tag of His-rTM₄₅₆-N₃ and followed by treatment with DBCO-functionalized liposome (DBCO-PEG₂₀₀₀-DSPE, 1%) prepared above in Tris-HCl buffer (Tris-HCl 20 mM, NaCl 150 mM, pH 7.4). DLS was used to monitor the integrity of the vesicles during and after the coupling reaction.

5.2.11 Protein Assay by Bradford Method

1ml purified rTM liposome conjugate from CL-6B column was collected in a acetone compatible tube, followed by adding 4ml cool acetone (-20 °C). The mixture was vortexed and incubates for 60 mins at -20 °C, followed by centrifuging 10 minutes at 13,000-15,000 × g. Then, supernatant was removed carefully and not dislodge the protein pellet. Next, the acetone was allowed to evaporate from the uncapped tube at room temperature for 30 mins. 1ml Tris-HCl buffer (20mM, pH 7.4) was added for the downstream process and vortexed thoroughly to dissolve protein pellet. Finally, the protein assay was performed as instruction manual of Bradford protein assay (Bio-Rad).

5.2.12 Stability Evaluation of Liposome during Click Conjugation and Liposomal rTM Conjugate

The stability of the liposomes during coupling reaction and the final liposomal-rTM₄₅₆ conjugate products were monitored by measuring fluorescent leakage in comparison to the same type of liposomes unmodified having encapsulated self-quenching concentrations of 5,6-carboxyfluorescein (85 mM) using FluoroMax-2 (ISA, NJ). Briefly, the lipid film composed of DSPC, cholesterol, mPEG₂₀₀₀-DSPE, DBCO-PEG₂₀₀₀-DSPE (2:1:5%:2% molar ratio) was swelled in the dark with 2.5 mL Tris-HCl buffer (Tris-HCl 20 mM, NaCl 150 mM, pH 7.4) containing 85 mM 5,6-Carboxyfluorescein (5,6-CF) to form the multilamellar vesicle suspension. The crude lipid suspension was extruded through polycarbonate membranes (Millipore sizes from 600 nm, 200 nm and 100 nm, successively) to produce small unilamellar vesicles with an average mean diameter of 120 ± 10 nm, as judged by DLS. Separation of the CF vesicles from non-entrapped CF was achieved by gel filtration chromatography, which involved passage through a 1.5×20 cm column of Sephadex G-50. Copper free click conjugation was conducted using the same procedure. Twenty μ L of reaction solution was taken and mixed with 1980 μ L of Tris-HCl buffer (pH 7.4), and then the fluorescent intensity was measured by using FluoroMax-2. A control experiment was conducted, in which recombinant TM with azide group was replaced with recombinant TM without azide group in equal amounts. To evaluate extent of fluorescence release upon disruption in the conjugation, 20 μ L of 0.5 % Triton-100 solution was added into sample above to release all entrapped CF molecules from the liposomes and fluorescence intensity was measured.

5, 6-carboxyfluorescein (CF) released from liposomes in PBS (pH 7.4) buffer at room temperature was measured over time. The excitation and emission wavelengths of 5, 6-CF were 497 and 520 nm, respectively. The variation of the fluorescent intensity with release time was calculated according to the equation below

$$\text{Fraction of CF remaining in liposomes} = 1 - F/F_0$$

where F is the fluorescent intensity measured at any time during the experiment and F_0 is the total fluorescent intensity measured after disrupting liposomes completely with 0.5% Triton X-100 in PBS (pH 7.4) buffer.

5.2.13 Catalytic Cofactor Activity Assay of rTM Derivatives by Protein C Activity Assay

Catalytic cofactor activity assay of rTM derivatives including His-rTM₄₅₆-N₃, rTM₄₅₆-N₃, rTM₄₅₆-Galactose, rTM₄₅₆-glycopolymer and liposome-rTM₄₅₆ Conjugates were analyzed by protein C assay. The activity of recombinant TM₄₅₆ was defined as moles of produced APC per min by given amounts of rTM₄₅₆ protein and rTM₄₅₆ conjugates in the presence of thrombin. All activations of protein C by rTM conjugates were performed for 60 mins in 20 mM Tris-HCl buffer, pH 7.4, 100 mM NaCl, 0.1% BSA, and 5 mM Ca²⁺ at 37°C. Typically, 20 nM thrombin and different rTM concentrations (1, 1.5, and 2 nM) were incubated in the assay buffer. After 60 mins, 20 μL antithrombin III (3 μg/μL) and 20 μL heparin (1000 IU/mL) were added into the solution to stop the reaction. The inhibition of protein C activation was completed within 5 min at room temperature. The produced APC was measured through hydrolysis of a

chromogenic substrate (Spectrozyme PCa) by comparing a standard curve, in which the concentration of APC to the rate of p-nitroanilide (pNA) formation was measured. The hydrolysis of Spectrozyme PCa was performed for 10 min in the assay buffer at 37°C, in which pNA was produced and the concentration measured by monitoring at $\lambda = 405$ nm with a spectrophotometer.

5.3 Result and Discussion

5.3.1 Synthesis of DBCO-PEG₄-CONH-Ph-Gal and Site-specific Galactose-Modification of His-rTM₄₅₆-N₃ via Copper-Free Click Chemistry

The DBCO functionalized galactose was synthesized by reaction between NHS activated DBCO and primary amine in the galactoses (Figure 5.2). The product was characterized by ¹H-NMR (Figure 5.3) and FTIR spectroscopy data (Figure 5.4). Finally, Mass spectrometry was used to detect the molecule weight. The produce was reconstituted in Solvent (50% acetonitrile, 50% water, and 0.1% formic acid) with 1.0µg/ml and load into ESI mass spectrometer by providing a peak at 850.23 (Figure 5.5), which fits the calculated molecule weight (849.92)

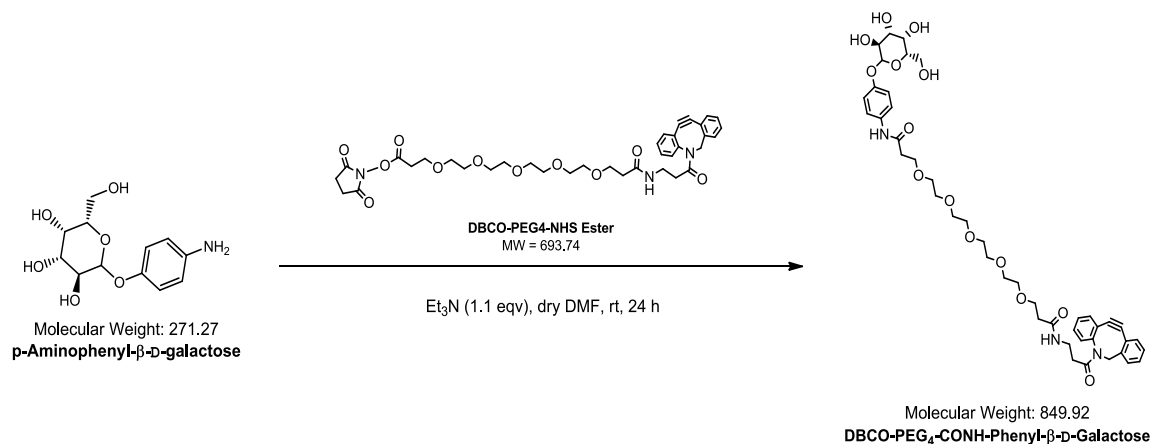


Figure 5.2 Scheme for Synthesis of DBCO-PEG4-CONH-Ph-Gal

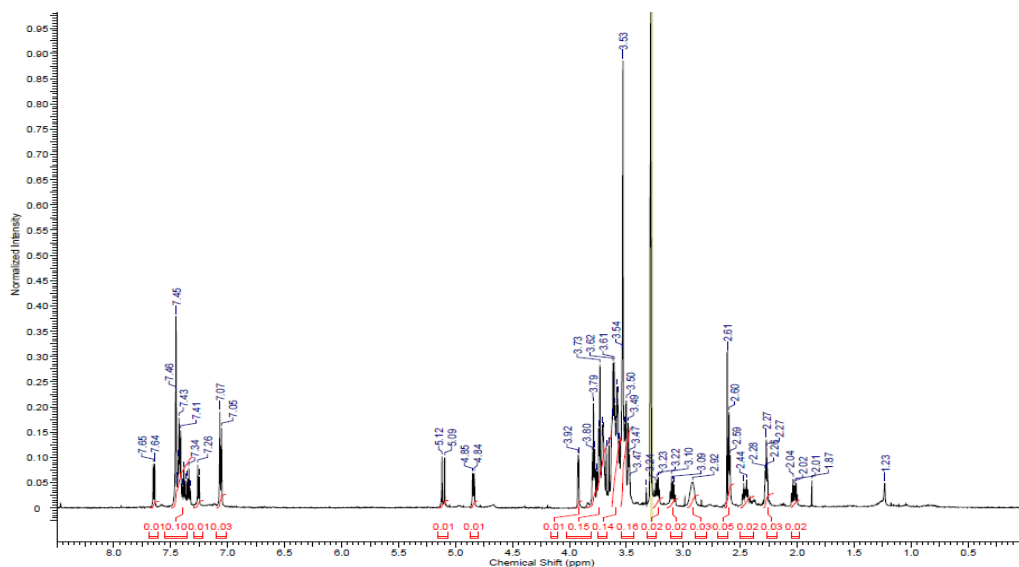


Figure 5.3 ¹H NMR Spectrum (CD₃OD/ D₂O) of DBCO-PEG₄-Phenyl-Galactose

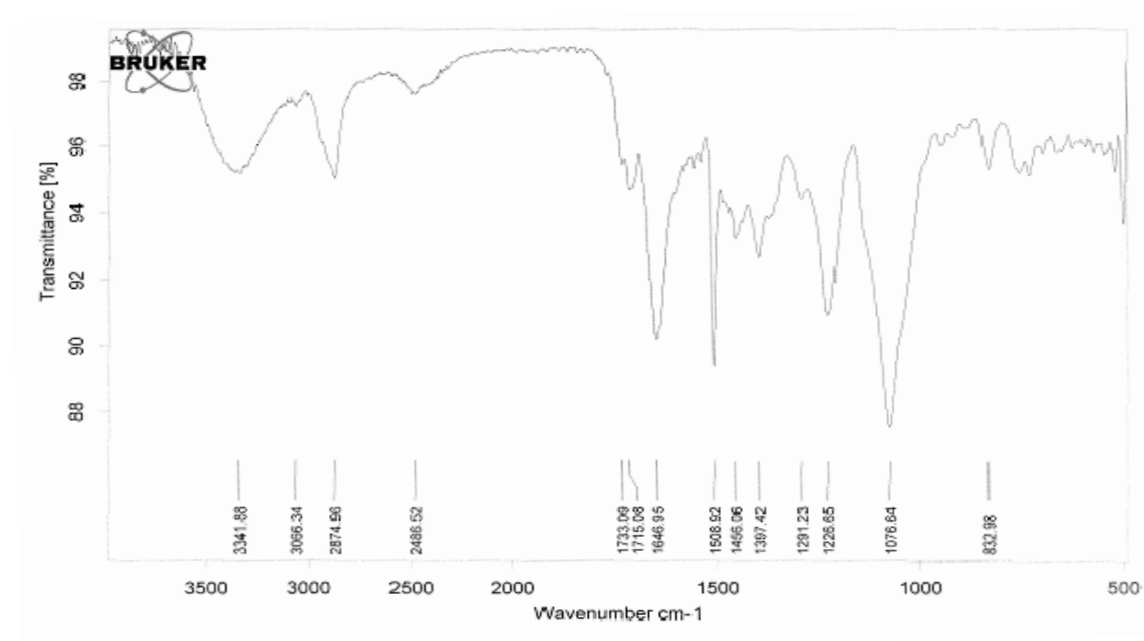


Figure 5.4 FTIR Spectrum (cm⁻¹) of DBCO-PEG₄-Phenyl-Galactose

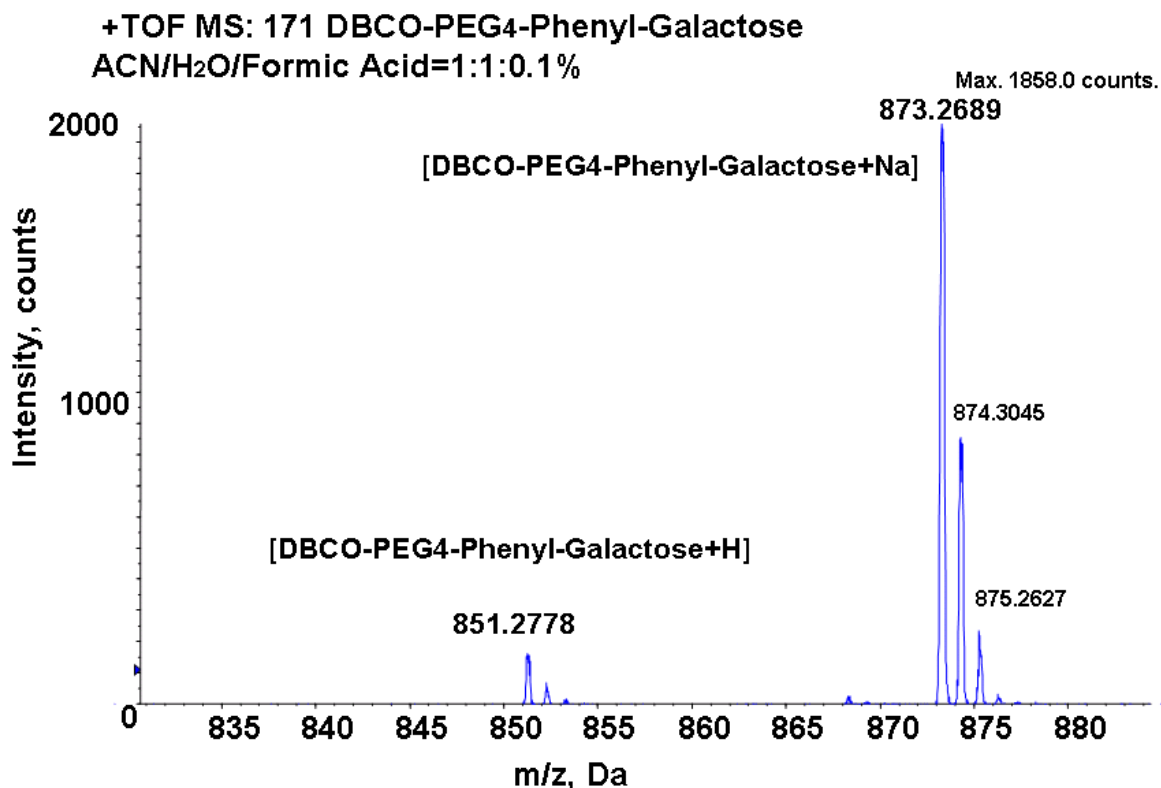


Figure 5.5 Mass spectrum of DBCO-PEG4-Phenyl-Galactose

Modification of proteins with carbohydrates has become a developing field in the enhancement of protein therapeutics [2, 18]. To test whether rTM₄₅₆-azide could be conjugated to a dibenzylcyclooctyne (DBCO) sugar derivative, our initial glyco-modification of the protein via copper-free click chemistry was carried out with a DBCO-containing galactose (DBCO-PEG₄-CONH-Ph-β-Gal), followed by glyco-staining and Coomassie blue staining to confirm the reaction product, respectively. rTM₄₅₆ without an azide group was used as a control for this experiment. The employment of the glyco-stain for carbohydrate detection allowed for the detection of attached carbohydrate based on a carbohydrate-specific periodic acid Schiff (PAS) staining method, in which the cis-diol sugar groups were oxidized to aldehydes and then subsequently reacted with Schiff reagent to yield a magenta band [19]. As shown in Figure 5.6, only the sample containing

His-rTM₄₅₆ with azide and DBCO-PEG₄-CONH-Ph-β-Gal (Lane 1) exhibited a magenta band whereas sample of His-rTM₄₅₆ without azide and DBCO-PEG₄-CONH-Ph-β-Gal (Lane 2, positive control) and sample of His-rTM₄₅₆ with azide and Phenyl-galactose without DBCO (Lane 3, negative control) showed no glyco-staining. Additionally, Coomassie blue staining for all proteins in the gel did not exhibited any new bands, since the molecular mass of the DBCO-PEG₄-CONH-Ph-β-Gal is only 849 Da, too small to alter protein migration pattern to be detected on SDS-PAGE. These results indicated the successful modification of His-rTM₄₅₆-azide with the galactose derivative via copper-free click chemistry.

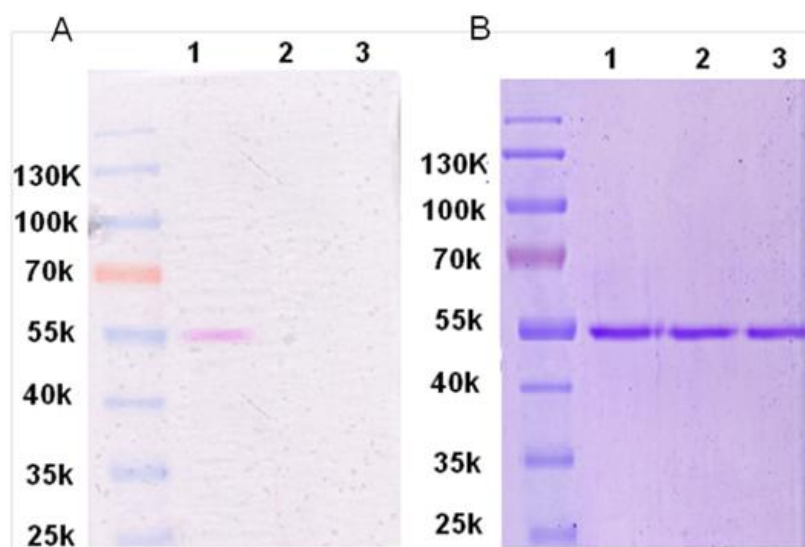


Figure 5.6 SDS-PAGE (12%) Characterization of Site Specific Glyco-Modification of rTM via Copper Free Click Chemistry (pH 8.0, room temperature (RT), 6 hrs): A. Glyco-staining, B. Coomassie blue staining: Lane 1: His-rTM-N₃ treated with DBCO-PEG₄-CONH-Ph-β-Gal; Lane 2 : His-rTM treated with DBCO-PEG₄-CONH-Ph-β-Gal; and Lane 3: His-rTM-N₃ treated with pNH₂-Ph-Gal.

5.3.2 Synthesis of DBCO-functionalized Glycopolymer and Site-Specific Glycopolymer-Modification of His-rTM₄₅₆-N₃ via Copper-Free Click Chemistry

Synthesis of DBCO-functionalized glycopolymer was conducted in two steps (Figure 5.7). First, lactose-based O-cyanate chain-end functionalized glycopolymer was synthesized from a lactose acrylamide derivative via cyanoxyl mediated free radical polymerization in our lab [17]. Then the glycopolymer was incubated with DBCO-PEG₄-Amine in bicarbonate buffer (pH 8.3) for 12h at room temperature to provide the DBCO functionalized glycopolymer with 70% yield. This product was characterized by ¹H NMR spectroscopy data (Figure 5.8).

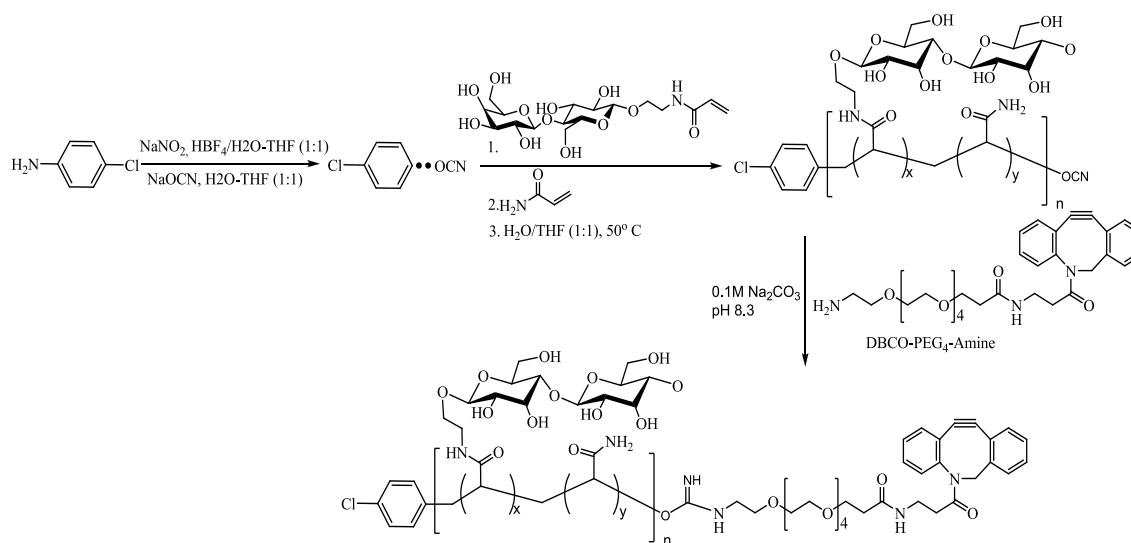


Figure 5.7 Synthesis of DBCO-Functionalized Glycopolymer

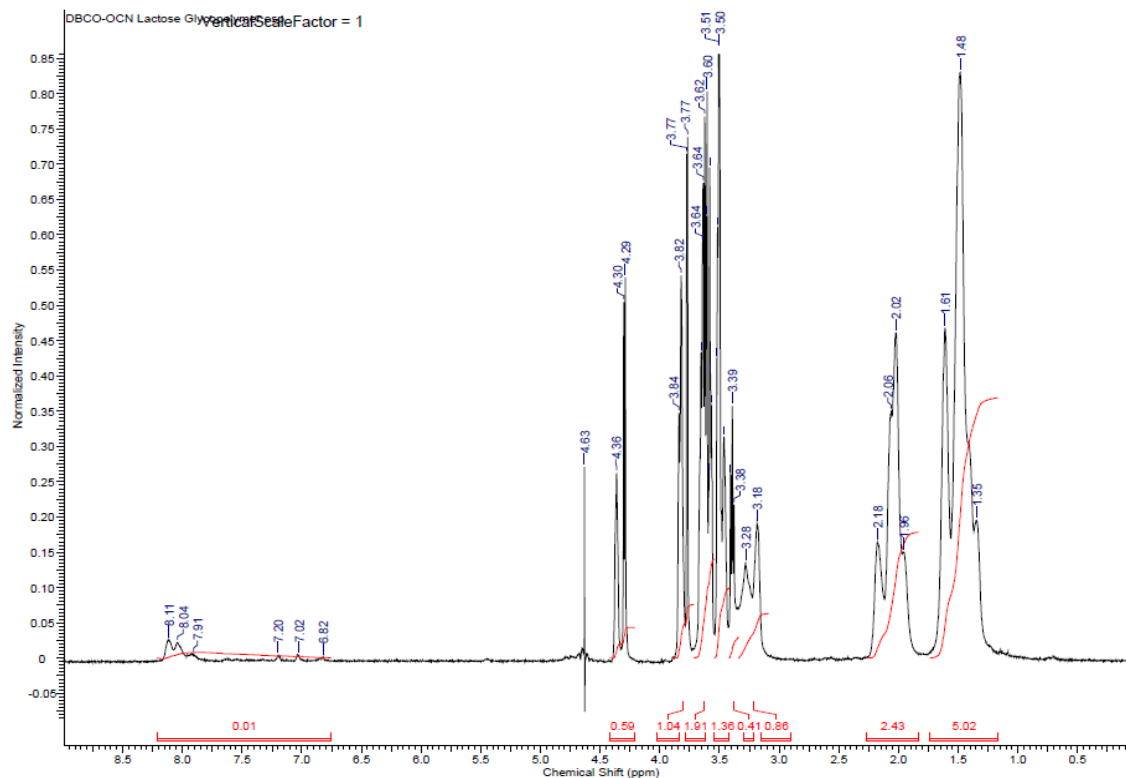


Figure 5.8 ^1H NMR Spectrum ($\text{CD}_3\text{OD}/\text{D}_2\text{O}$) of DBCO-Functionalized Glycopolymer

Next, a site-specific glycopolymer modification of the recombinant TM was investigated using a DBCO-PEG₄-Glycopolymer via copper-free click chemistry as indicated above. SDS-PAGE was used to separate and identify the conjugate, followed by glyco-staining and Coomassie blue staining, respectively. As shown in Figure 5.9, only the sample including His-rTM₄₅₆-N₃ (with azide) (Lane 1) showed a magenta band of high molecular weight (>130 kDa) while the His-rTM₄₅₆ (without azide) treated with DBCO-PEG₄-Glycopolymer (Lane 2, positive control) and His-rTM₄₅₆-N₃ (with azide) treated with glycopolymer without DBCO (Lane 3, negative control) exhibited no glyco-staining (Figure 5.9A). Coomassie blue staining (Figure 5.9B) showed the same high molecular weight band that appeared on the glyco-staining gel on Lane 1 containing His-

rTM₄₅₆-N₃ (with azide) and DBCO-PEG₄-Glycopolymer, and the protein band at ~51 kDa corresponding to unreacted His-rTM₄₅₆ azide.

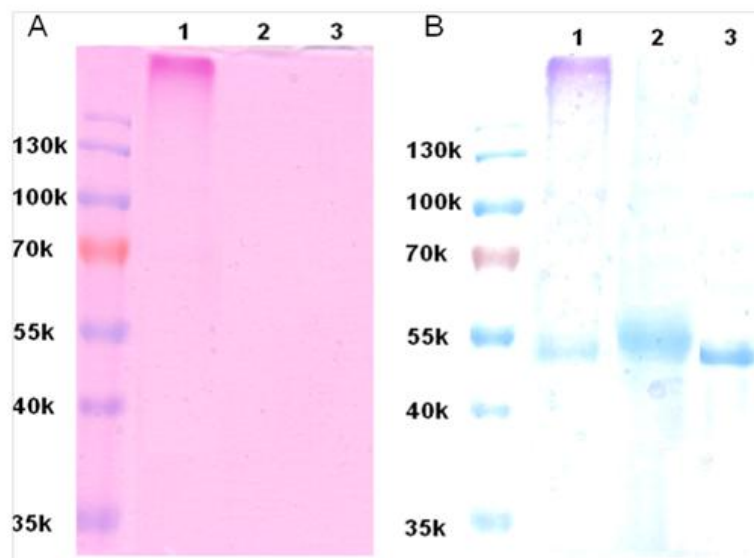


Figure 5.9 SDS-PAGE (12%) Characterization of Site-Specific Glycopolymer-Modification of rTM via Copper Free Click Chemistry (pH 8.0, RT, 6 hrs): A. Glyco-staining, B. Coomassie blue staining: Lane 1: His-rTM-N₃ treated with DBCO-PEG₄-Glycopolymer; Lane 2: His-rTM treated with DBCO-PEG₄-Glycopolymer; and Lane 3: His-rTM-N₃ treated with glycopolymer (without DBCO).

The molecular weight achieved for the glycopolymer-protein conjugate was higher than anticipated on the SDS-PAGE gel. This in part could be due to the fact that the glycopolymer attached is a long chain molecule consisting of lactose and amide side chains with very little charge, which may hamper the conjugate's ability to move within the electric field during electrophoresis. This phenomena has been observed in previous report when using glycopolymer to modify streptavidin [20]. Neither the positive nor did the negative controls exhibit the formation of any new bands after Coomassie blue staining. These results indicated successful glycopolymer modification of the rTM₄₅₆-N₃ via copper-free click chemistry. Specifically, the reaction between two

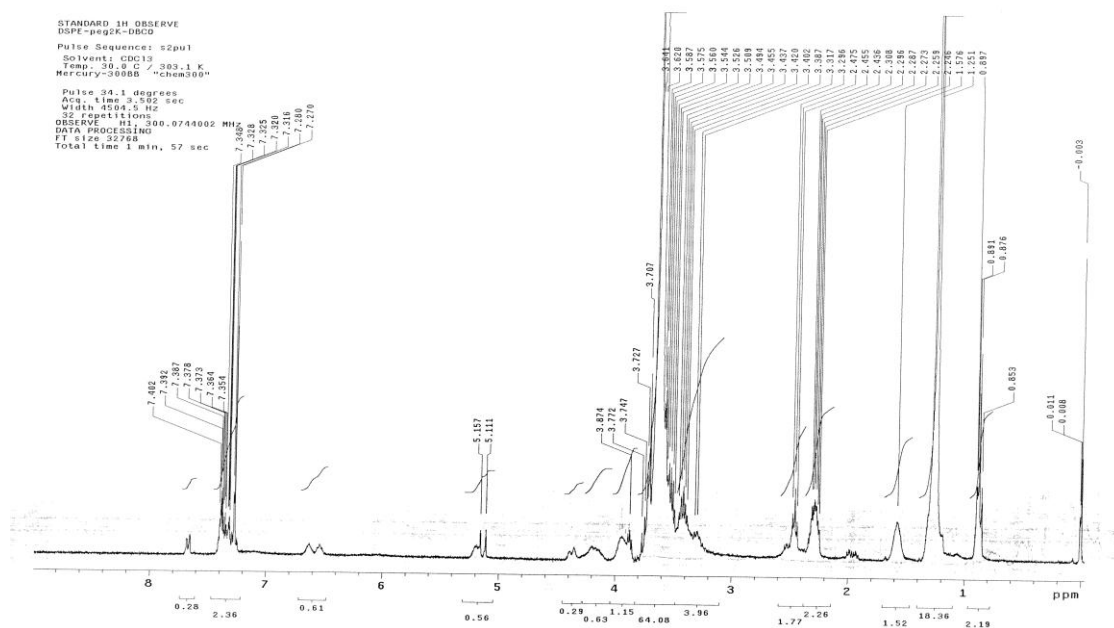


Figure 5.11 ^1H NMR Spectrum ($\text{CD}_3\text{OD}/\text{D}_2\text{O}$) of DSPE-PEG₂₀₀₀-DBCO

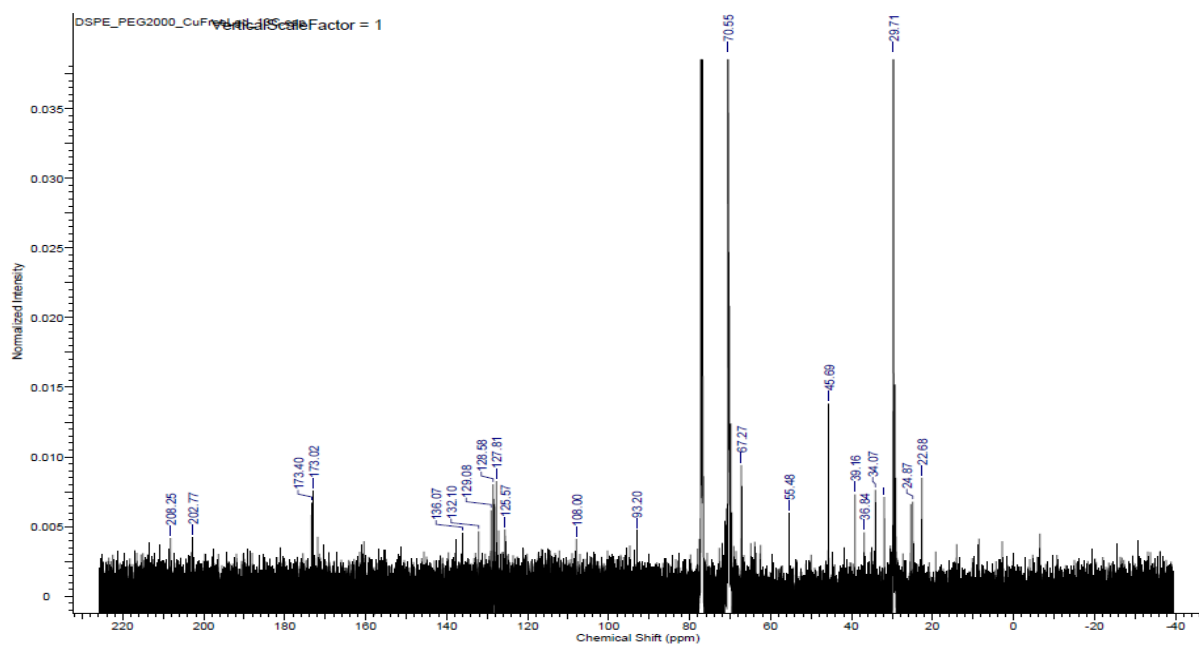


Figure 5.12 ^{13}C -NMR Spectrum ($\text{CD}_3\text{OD}/\text{D}_2\text{O}$) of DSPE-PEG₂₀₀₀-DBCO

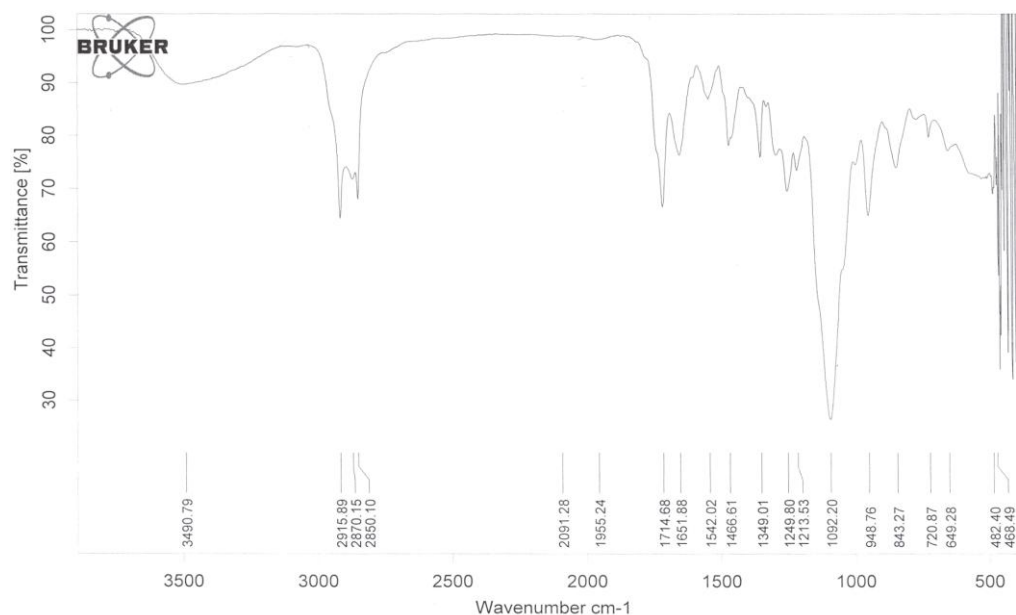


Figure 5.13 FTIR Spectrum of DSPE-PEG2000-DBCO

With the anchor lipid (DSPE-PEG₂₀₀₀-DBCO) in hand, copper free click conjugation of His-rTM₄₅₆-N₃ to anchor lipid was investigated to explore conjugation condition. To a solution of recombinant His-TM₄₅₆-N₃, anchor lipid was added, and then the mixture was stirred for 9hrs at room temperature. The efficiency of click conjugation between anchor lipid and recombinant His-TM₄₅₆-N₃ was evaluated given amount of His-rTM₄₅₆-N₃ and anchor lipid over time. This conjugation was monitored by 12% SDS-PAGE gel and visualization of protein bands by Coomassie blue staining. At the same time, western blot was used to confirm the conjugate. Control positive control (His-rTM₄₅₆-N₃ and lipid without DBCO group) and negative control (His-rTM₄₅₆ and anchor lipid with DBCO group) were conducted in the same procedure was conducted by incubation of same amount of anchor lipid into rTM₄₅₆ without azide group solution of Tris-HCl buffer. As result, a new broad band was observed in the conjugation while

there was no any new band in the positive control and negative control experiment by conducting His-rTM₄₅₆ without azide group in its C terminal. According the SDS-PAGE and Western Blot above, the conjugation was ended in 6 hours and there was no more conjugate produced with continuously increasing incubation time to 9 hrs. Based on the SDS-PAGE and Western blot (Figure 5.14), about 90% of rTM₄₅₆ was modified by anchor lipid by copper click conjugation.

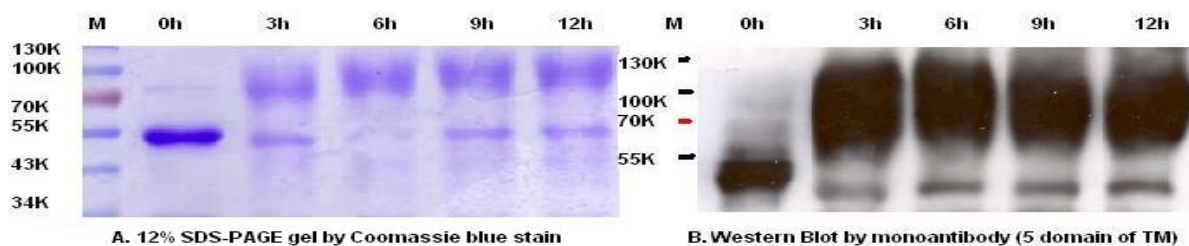


Figure 5.14 SDS-PAGE and Western Blot for Monitoring Conjugation of His-rTM-Azide with DSPE-PEG₂₀₀₀-DBCO (lipid anchor only)

5.3.4 Synthesis of Liposomal rTM₄₅₆ Conjugates via Copper-Free Click Chemistry

In order to make a membrane mimetic TM conjugate, we then investigated the selective click chemistry conjugation of the rTM₄₅₆-N₃ onto alkyne-PEG functionalized liposomes. Conventional methods in the preparation of surface functionalized liposomes involve the initial synthesis of the key lipid-ligand conjugate, followed by formulation of the liposome with other lipid components. In this liposome formulation method, some of the valuable ligands inevitably face the enclosed aqueous compartment and thus become unavailable for their intended interaction with their target molecules. Therefore, in a liposome formulation for reagent delivery, it is unrealistic if the targeting ligand is only available in minimal amounts, which may hinder the ability of the carrier to reach its destination and to properly interact with its target especially when multivalent binding is necessary. Furthermore, lipid-ligand conjugates may have poor solubility and stability in

aqueous solvent, or are incompatible with various manufacturing processes. In this study, a chemically selective post-modification approach was investigated to generate the proposed rTM-liposome conjugate. Briefly, liposomes bearing the anchor lipid DBCO-PEG₂₀₀₀-DSPE were prepared, followed by incubation with His-rTM₄₅₆-N₃ in a Tris-HCl buffer (Tris-HCl 20 mM, NaCl 150 mM, pH 7.4). The click conjugation was then conducted at room temperature for up to 9 h under very gentle shaking. The unreacted His-rTM₄₅₆-N₃ was then removed by gel filtration (1.5 x 20 cm column of Sephadex G-50) to afford the targeted His-rTM₄₅₆-liposome conjugate. SDS-PAGE and Western blotting were used to monitor the conjugation progress. As shown in Figure 5.15 (A and B), the His-rTM₄₅₆-PEG₂₀₀₀-DSPE formed was observed as a new band after conjugation, while neither the positive nor the negative control experiments exhibited any new bands. The His-rTM₄₅₆-PEG₂₀₀₀-DSPE conjugate was further confirmed by BaCl₂/I₂ staining which visualizes the PEG molecules of the conjugate (Figure 5.15C), whereas both positive (His-rTM₄₅₆-N₃ and liposome without anchor lipid, Figure 5.15D) and negative (His-rTM₄₅₆ and liposome with anchor lipid, Figure 5.15E) control conjugations did not show His-rTM₄₅₆-PEG₂₀₀₀-DSPE conjugate formation. These results indicated successful liposomal modification of the rTM-N₃ via copper-free click chemistry. As in the same procedures above, the targeted rTM₄₅₆-liposome conjugate was synthesized by releasing the His tag of His-rTM₄₅₆-N₃ and followed by treatment with liposomes bearing the anchor lipid DBCO-PEG₂₀₀₀-DSPE in Tris-HCl buffer (Tris-HCl 20 mM, NaCl 150 mM, pH 7.4). As shown in Figure 6.15F, Western blotting confirmed the new band formed upon the rTM₄₅₆-N₃ reacted with the liposome.

The conjugation efficiency of the click conjugation was evaluated by a given amount of rTM₄₅₆-N₃ and fixed anchor lipid (DCBO-PEG₂₀₀₀-DSPE) concentration in the liposome (2% of the lipids in liposome). The grafted amount of rTM₄₅₆-N₃ onto the liposome was calculated after gel filtration purification (CL6B, GE Healthcare) by detecting unreacted rTM₄₅₆-N₃ in the buffer and rTM₄₅₆-PEG₂₀₀₀-DSPE formed, respectively, in which DCBO-PEG₂₀₀₀-DSPE was in excess to rTM₄₅₆-N₃. Acetone was used to precipitate proteins allowing for the physical separation of protein pellet from the liposomal solution. The pellet was then redissolved in Tris-HCl buffer (pH 8.0) and detected by the Bradford protein assay (Bio-Rad). As a result, the conjugation yield was ~60% by detecting the rTM-liposome conjugate formed and ~68% by detecting the unreacted rTM₄₅₆-N₃ left, respectively.

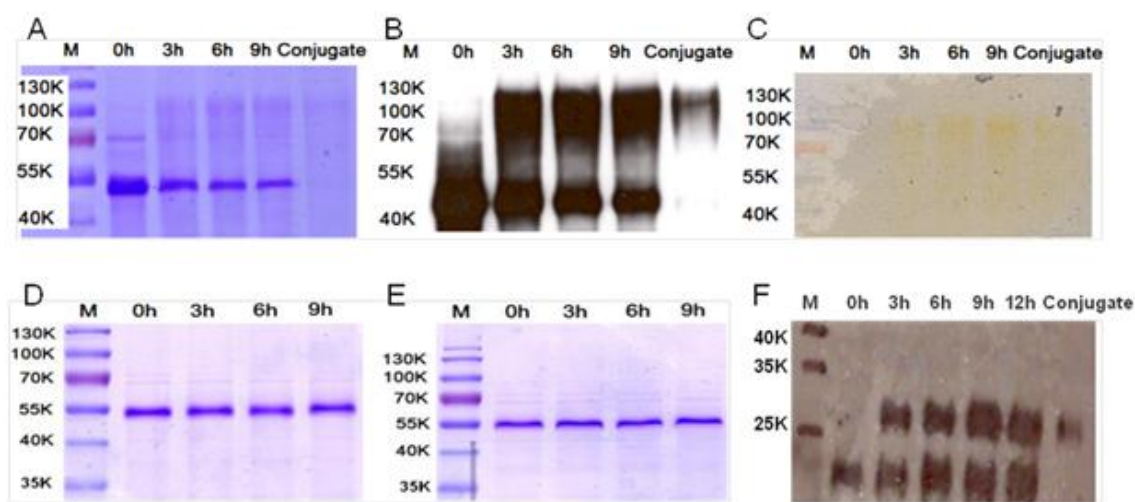


Figure 5.15 SDS-PAGE (12%) Characterization of Site Specific Liposomal Modification of His- rTM-N3 via Copper Free Click Chemistry (pH 8.0, RT): A. Coomassie blue staining; B. Western blot; C. barium chloride/iodine staining; D. positive control: His-rTM₄₅₆-N₃ treat with liposome without anchor lipid; E. negative control: His-rTM₄₅₆ and liposome with anchor lipid DBCO-PEG₂₀₀₀-DSPE, F. Western blot for rTM₄₅₆-N₃ treated with liposome with anchor lipid DBCO-PEG₂₀₀₀-DSPE

5.3.5 Stability Evaluation of Liposome during Click Conjugation and Liposomal rTM Conjugate

Dynamic light scattering (DLS) was used to monitor the reaction and verify the integrity of the liposome during and after the click conjugation reaction. As shown in Figure 5.16, there was no significant change in the size of the vesicles during and after conjugation reaction. The average of liposome size increased from 118 ± 5 nm (Figure 5.16A) to 140 ± 5 nm (Figure 5.16B). After separation of the rTM-liposome conjugate from the reaction solution, the rTM-liposome conjugate did not show size change (Figure 5.16C). Furthermore, there was no size change of control experiment (119 ± 10 nm) in the presence of rTM₄₅₆ without azide group (Figure 5.16D). This result indicated that the liposomes were intact during the reaction and purification processes.

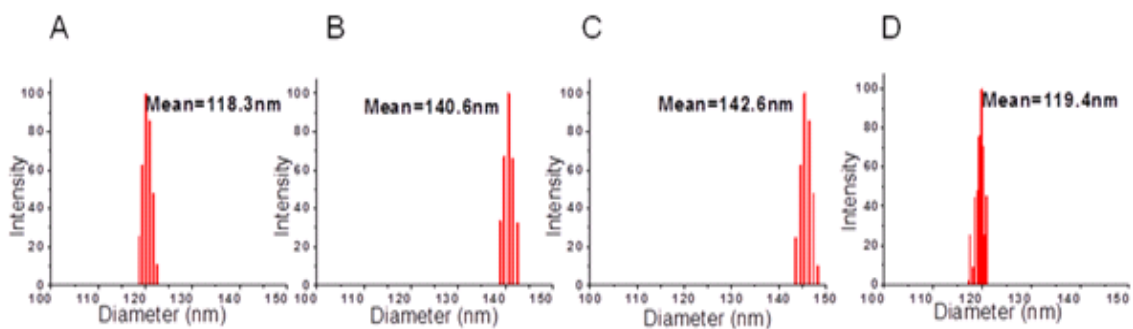


Figure 5.16 DLS monitoring of liposome stability during copper free click chemistry conjugation between liposome (DBCO-PEG₂₀₀₀-DSPE, 2%) and rTM₄₅₆-N₃ (without His-tag): A. before conjugation; B. after conjugation; C. purified rTM-liposome conjugate; D. control: liposome (DBCO-PEG₂₀₀₀-DSPE, 2%) treated with rTM₄₅₆ (without N₃)

To test whether the conjugation could provoke leakage in the liposomes during the reaction and the stability of liposomal conjugate, fluorescent dye leakage of the same type of liposomes having encapsulated self-quenching concentrations of 5,6-carboxyfluorescein (5,6-CF, 85 mM) was monitored using FluoroMax-2 (ISA) spectrometer. Based on the fluorescence quenching determinations (Figure 5.17A) and rTM-liposome conjugate releasing assay (Figure 5.17B), we demonstrated that no apparent leakage was triggered by the conjugation reaction compared to the liposomes incubated in the presence of rTM₄₅₆ without azide group. This fluorescence intensity unchanging indicated that no liposome disruption occurred during the click conjugation even after 12 h. In addition, the conjugation of the rTM to the liposome surface may have enhanced the liposome stability as it exhibited significantly slower dye release between 14 to 21 days as compared to the control liposome treated with rTM without azide (Figure 5.17B). These results indicated the compatibility of the copper-free click chemistry for rTM-liposome formation, and the rTM-liposome conjugate was quite stable and could be used for further activity evaluation

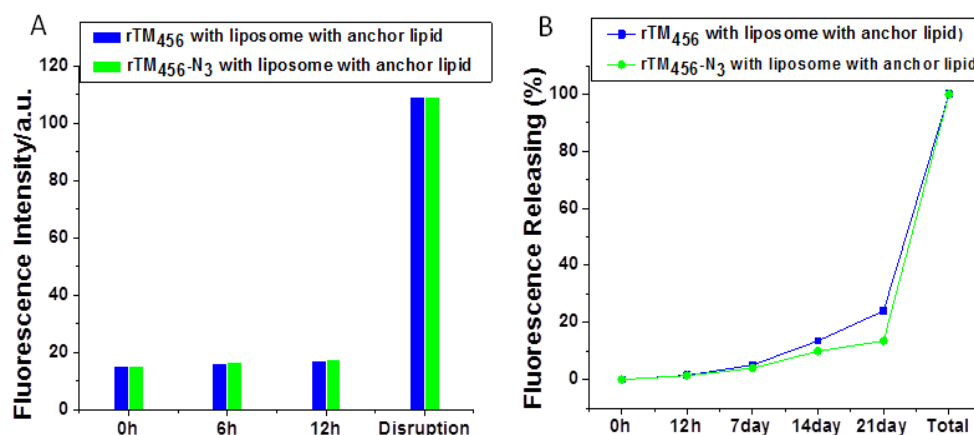


Figure 5.17 Evaluation of Stability of Liposome during Click Conjugation Reaction (A) and Stability of rTM-Liposome Conjugate (B) by Monitoring Fluorescent Dye 5,6-CF

Releasing from Liposomes. Disruption of liposomes was conducted by adding PBS buffer containing 0.5% Triton X-100 surfactant into the liposome solutions.

5.3.6 Catalytic activity assay of rTM conjugates

In this study, protein C activation activities of the rTM conjugates were evaluated. The activity of rTM₄₅₆ was defined as moles of produced activated protein C per min by given amounts of rTM₄₅₆ and rTM₄₅₆ conjugates in the presence of thrombin. All protein C activation assays by rTM₄₅₆ and rTM₄₅₆ conjugates were performed for 60 mins in a buffer with 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% BSA, and 5 mM Ca²⁺ at 37 °C, as previously reported [21]. The catalytic activity of rTM₄₅₆ and rTM₄₅₆ conjugates were evaluated via the activation of varying human protein C concentration by rTM₄₅₆ and rTM₄₅₆ conjugates in the presence of thrombin and calcium. The rate of protein C activation was linear with time until at least ~20% of the protein C was activated. Reaction conditions (TM concentration or incubation time) were adjusted so that less than 10% of the protein C was activated to ensure that the amount of activated protein C by TM was in the linear range. As shown in Table 5.1, there was no activity change upon glyco-modification of rTM₄₅₆ with either galactose or lactose-containing glycopolymer. However, rTM-liposome conjugates had a 2-fold higher k_{cat}/K_M value than that of either His-TM₄₅₆-N₃ or rTM₄₅₆-N₃, even though there was no apparent change of k_{cat} . This change in k_{cat}/K_M mainly was due to a decreased K_M value, which represents the affinity of rTM for protein C. This result was consistent with a previous report of increased k_{cat}/K_M values due to an higher affinity of rTM for protein C [22]. This increased affinity may result from using liposome as a platform, which mimics endothelial cell lipid

membrane and has a beneficiary effect on the conformation of the conjugated rTM for enhancing thrombin and protein C binding.

Table V. Protein C Activation Activity of rTM and its Conjugates via Copper Free Click Chemistry

	Full TM*	His-rTM-N ₃	rTM-N3	rTM-Gal	rTM-GP	rTM-liposome
K_M (μ M)	0.60 \pm 0.15	0.80 \pm 0.2 (0.90 \pm 0.2)	0.90 \pm 0.2 (1.0 \pm 0.5)	0.95 \pm 0.22	0.87 \pm 0.15	0.33 \pm 0.17
k_{cat} (min^{-1})	0.20 \pm 0.03	0.28 \pm 0.05 (0.22 \pm 0.05)	0.24 \pm 0.04 (0.16 \pm 0.05)	0.22 \pm 0.12	0.18 \pm 0.17	0.23 \pm 0.17
k_{cat}/K_M ($\text{min}^{-1}.\mu\text{M}^{-1}$)	0.37 \pm 0.14	0.40 \pm 0.15 (0.26 \pm 0.1)	0.29 \pm 0.11 (0.16 \pm 0.05)	0.25 \pm 0.14	0.18 \pm 0.11	0.71 \pm 0.32

*commercial full TM of human, Gal: galactose, GP: glycopolymer. The data in parentheses are from Chaikof et al. [10]

5.4 Conclusion

We demonstrated here the first example of biomimetic TM conjugates that were synthesized by site-specific conjugation of recombinant TM at the C-terminal with glycopolymer and liposome via copper-free click chemistry. The protein conjugation method presented here has distinct advantages over traditional methods. First, the site-specific, chemo- and bio-orthogonal conjugation provides a simple and convenient route to uniform protein conjugation in short time and good yields. Second, mild conjugation conditions and low temperatures minimize the chance of protein denaturation. The rTM-liposome conjugate showed a 2-fold higher k_{cat}/K_M value for protein C activation than that of the rTM₄₅₆, which indicated that the biomimetic lipid membrane has a beneficiary

effect on the rTM's activity. Continued studies of in vitro and in vivo antithrombotic activity of these TM conjugates and their pharmacokinetic properties are under investigation. Overall, the proposed glyco-engineering of recombinant TM with glycopolymer and membrane-mimetic re-expression of recombinant TM onto liposome provide a rational design strategy for facilitating studies of membrane glycoprotein TM functions and generating a TM-based antithrombotic drug.

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CHAPTER VI

**AZIDE-REACTIVE LIPOSOME FOR CHEMOSELECTIVE AND
BIOCOMPATIBLE LIPOSOME IMMOBILIZATION AND GLYCO-
LIPOSOMAL MICROARRAY FABRICATION**

(Partial results are from Ma, Y., Zhang, H. L., Sun, X-L. *Langmuir*. 2011, 27, 13097-13103 and Ma, Y., Zhang, H. L., Sun, X-L. *Bioconjug. Chem.* 2010, 21, 1994-1999)

6.1 Introduction

Immobilization of liposome onto solid surface has shown a great potential in biological and biomedical research and applications [1]. This discipline has been inspired by that liposome structurally retains the properties inherent in natural lipid membranes, and functionally can serve as model of biomembrane and can encapsulate both hydrophobic and hydrophilic compounds such as drug and gene for delivery applications [2]. For example, immobilized liposomes have been investigated as model systems presenting lipid membranes for bioseparation [3], biosensor [4], and nanobioreactor [5] applications. Recently, immobilized liposomes onto a biomedical device have been considered as a potential local drug delivery system, which release drug immediately to

the environment surrounding the device, and reduce the toxic effects on other organisms and thus enhance the therapeutic effect of the drug [6]. In addition, liposome microarray has been explored recently for applications in membrane biophysics, biotechnology, and colloid and interface science [7].

Surface-immobilized liposomes can be fabricated through either noncovalent such as bio-affinity interaction or covalent bond formation by synthesizing anchor group modified liposomes. Conventionally, the anchor group modified liposomes are prepared by direct liposome formation method, in which the anchor lipid is synthesized first and followed by formulation of the liposome with all other lipid components. In this direct liposome formation method, however, some anchor-lipid conjugates may have limited solubility and stability in solvent, or are incompatible with various stages of preparation, or even may have difficulty to form liposome due to the loss of its amphiphilic property. It is well known that the shape of the self-assembled liposomes may be influenced by the nominal geometric parameters of its molecule such as polar head surface, tail volume and chain length [8]. Alternatively, anchor group modified liposomes can be synthesized by chemical modification of reactive preformed liposomes [9]. Variable successes using amide [10] or thiol-maleimide coupling [11] as well as by imine [12] or hydrazine linkage [13] have been reported. However, non-chemoselective, harsh reaction conditions and low efficiency of most these methods limited their practical applications.

Azide-based ligation reactions have been extensively explored for highly sensitive and biocompatible bioconjugation [14-16], polymer and materials science [17, 18] and drug discovery [19, 20]. Specifically, the azide is a versatile bioorthogonal chemical reporter. Its small size and stability in physiological settings have enabled

azide-functionalized metabolic precursors to hijack the biosynthetic pathways for numerous biomolecules, including glycans [21], proteins [15, 22], lipids [23] and nucleic acid-derived cofactors [24] and therefore can afford a variety of azide-containing biomolecules for biomedical applications. Three reactions have been reported for tagging azide-labeled biomolecules. One of these, the Staudinger ligation capitalizes on the selective reactivity of phosphine and azide to form an amide bond [14, 25, 26]. The other two involve the reaction of azide with alkyne to give triazole, a process that is typically very slow under ambient conditions. The Cu(I)-catalyzed azide-alkyne cycloaddition also known as "click chemistry", accelerates the reaction by use of a toxic copper catalyst [27, 28]. The copper may residue inside of the liposomes and thus cause problem in clinical application. Recently, the strain-promoted [3 + 2] cycloaddition removes the requirement for cytotoxic copper by employing cyclooctynes that are activated by ring strain [29, 30]. However, two triazole regioisomers forms during the conjugation, which affords complicated products without controlling [31]. Most recently, we have demonstrated that Staudinger ligation of triphenylphosphine-carrying liposome with azide-containing biomolecules as a chemoselective liposome surface functionalization approach [32]. The high specificity, high yield, biocompatible and the lack of residual copper reaction condition natures of the Staudinger ligation approach make it an attractive alternative to all currently used protocols for liposome surface functionalization. Herein, we investigated expanded application of this azide reactive liposome for efficient and chemical selective liposome surface immobilization and microarray fabrication applications (Figure 6.1). Specifically, microarray of liposome carrying triphenylphosphine onto azide-modified glass slide and further glyco-modification with

azide-containing carbohydrate provides a cytomimetic glycoarray, which may find important biomedical applications such as studying carbohydrate-protein interaction and toxin and antibody screening and so on.

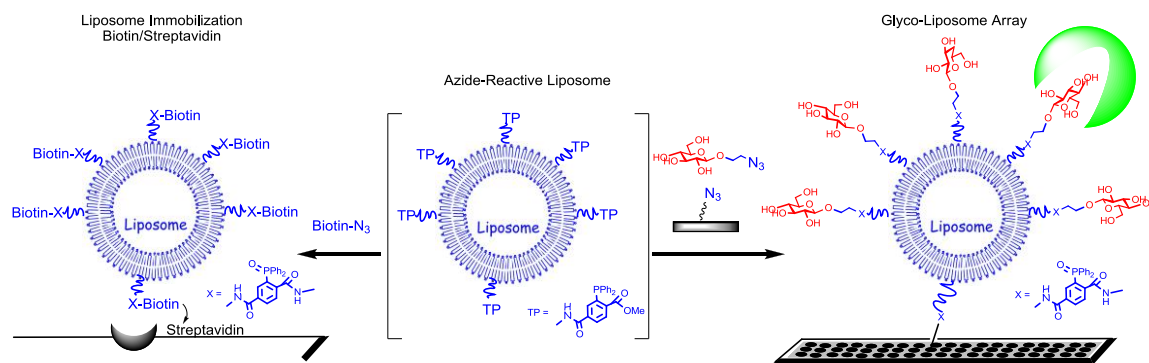


Figure 6.1 Illustration of Chemically Selective and Biocompatible Liposome Surface Functionalization and Immobilization via Staudinger Ligation

6.2 Experimental

6.2.1 Materials and Methods

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)₂₀₀₀] (ammonium salt) (DSPE-PEG₂₀₀₀), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)₂₀₀₀] (ammonium salt) (DSPE-PEG₂₀₀₀-biotin), 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (DPPE-NBD) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol, dicyclohexylcarbodiimide (DCC), diphenylphosphino-4-methoxycarbonylbenzoic acid, hexaethylene glycol, toluene sulfonyl chloride, imidazole, sodium azide, N-hydroxysuccinimidobiotin, N,N-dimethylformamide were purchased from Sigma (USA). All other solvents and reagents

were purchased from commercial sources and were used as received, unless otherwise noted. Deionized water was used as a solvent in all experiments.

Instrumental analysis. Dynamic Light Scattering was measured with 90plus particle size analyzer (Brookhaven Ins. Co., USA). Atomic force microscopes were carried out using PicoPlus 3000 (Molecular Imaging, USA) and Fluorescence imaging were obtained by Typhoon 9410 Variable Mode Imager (Amersham Biosciences, USA).

6.2.2. Synthesis of Anchor Lipid DSPE-PEG₂₀₀₀-Triphenylphosphine (1)

DSPE-PEG₂₀₀₀-NH₂ (100 mg, 35.8 μ mol) was dissolved in 20 mL of CH₂Cl₂, and 0.2 mL of triethylamine was added. After stirring for 30 min at room temperature, a solution of succinimidyl 3-diphenylphosphino-4-methoxycarbonylbenzoate (33 mg, 71.6 μ mol) in 50 mL of CH₂Cl₂ was added. The reaction mixture was stirred at room temperature for 24 h and then concentrated under vacuum to give a residue, which was purified by silica gel chromatography with chloroform/methanol (4:1, v/v) to afford product 1 (32 mg, 28.5 %). ¹H NMR (CDCl₃, 300 MHz) 8.06 (m, 1H), 7.79 (m, 1H), 7.44 (m, 1H), 7.66 (m, 2H), 7.52-7.42 (m, 2H), 7.28-7.34 (m, 8H), 6.64 (m, 1H), 5.19 (s, 1H), 4.34-4.20 (m, 3H), 3.95-3.80 (m, 3H), 3.80-3.50 (br. S, 44H, O-CH₂-CH₂-O), 3.40-3.20 (m, 3H), 2.28 (br.s, 4H), 1.52 (br.s, 4H), 1.36-1.20 (s, 32H), 0.89 (t, J, 6.9 , 6H), ³¹P NMR (CDCl₃, 121 MHz) : -2.7.

6.2.3. Synthesis of Azidoethyl-Tetra (ethylene glycol) Ethylamino Biotin (2)

Triethylamine (0.03 mL, 0.02 mmol) was added to a solution of amino-11-azido-3, 6, 9-trioxaundecane [20] (54 mg, 0.176 mmol) in DMF (3.5 mL). After the solution was stirred for 30 min, a solution of N-hydroxysuccinimidobiotin (50 mg, 146 μ mol) was added. The reaction mixture was stirred for 12 h at room temperature and then concentrated under vacuum to give a residue, which was purified by silica gel column chromatography using acetone: hexane (4:1, v/v) as eluent to afford 2 (41 mg, 44%). ^1H NMR (CD_3OD , 300 MHz) : 6.75 (br. S, 1H), 6.75 (br. S, 1H), 6.52 (br. S, 1H), 5.88 (br. S, 1H), 4.51 (m, 1 H, -CH-1-Biotin), 4.32 (m, 1 H, -CH-4-Biotin), 3.70-3.63 (m, 16 H, -O-($\text{CH}_2\text{CH}_2\text{O}$)₄-PEG), 3.56 (m, 2 H, -O- $\text{CH}_2\text{CH}_2\text{-N}_3$), 3.39 (m 4 H, - $\text{CH}_2\text{-NH}$ and -O- $\text{CH}_2\text{CH}_2\text{-N}_3$), 3.24 (m 1 H, -CH-3-Biotin), 2.92 (dd, 1 H, J = 4.8, 12.8 Hz, -CH-2a-Biotin), 2.71 (m 1 H, -CH-2b-Biotin), 2.23 (t, 1 H, J = 7.6 Hz, - $\text{CH}_2\text{CO-Biotin}$), 1.76-1.63 (m, 4 H, - $(\text{CH}_2)_2\text{-Biotin}$), 1.50-1.40 (m, 2 H, - $(\text{CH}_2)\text{-Biotin}$).

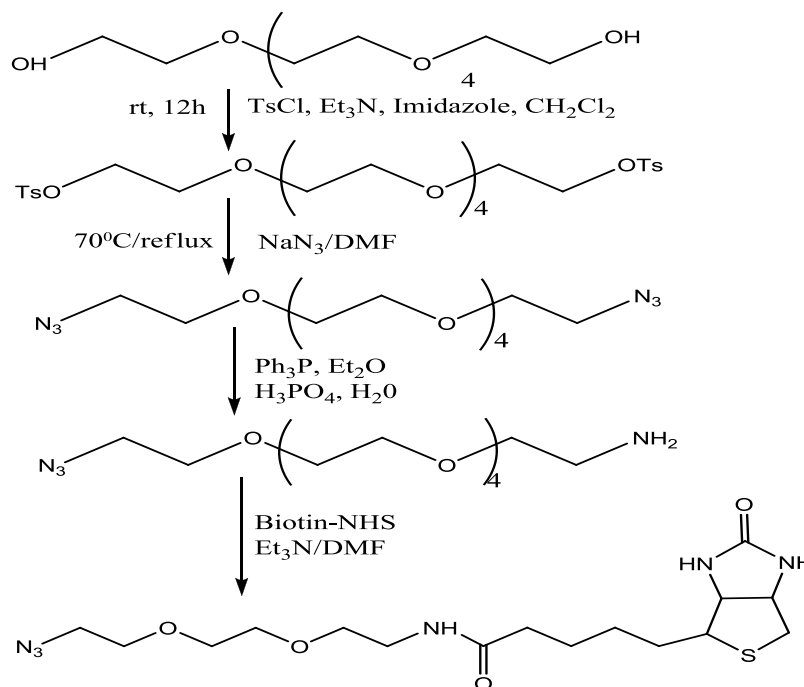


Figure 6.2 Scheme for Synthesis of Amino-11-Azido-3, 6, 9-Trioxaundecane

6.2.4 Preparation of Biotin-Liposome via Staudinger Ligation

First, triphenylphosphine functionalized liposome was prepared. DSPC and cholesterol at 2:1 mol ratio were used as the major components of all liposome. For liposome biotinylation, 0.5 mol % of anchor lipid DSPE-PEG₂₀₀₀-triphosphine was doped. To visualize the liposome immobilized onto the solid surface, all kinds of liposome were incorporated with DPPE-NBD (0.5 mg, 0.6 mol %). In detail, the mixture of lipids was first dissolved in chloroform. The solvent was gently removed on an evaporator under reduced pressure to form a thin lipid film on the flask wall and kept in a vacuum chamber overnight. Then, the lipid film was swelled in the dark with 2.5 mL PBS buffer (pH 7.4), followed 10 freeze-thaw cycles of quenching in liquid N₂ and then immersed in a 50 °C water-bath to form multilamellar vesicle suspension. Finally, the crude lipid suspension was extruded through polycarbonate membranes (pore size 600, 200, and 100 nm, gradually) at a 60 °C to afford small unilamellar vesicles.

Next, triphenylphosphine functionalized liposome incubated with biotin-PEG₆-azide to provide biotinylated liposome. In detail, to 2.5 mL of triphosphine-liposome in PBS (pH 7.4) above, 1 mL of biotin-PEG₆-azide (30 mg, 56 μmol) in PBS (pH 7.4) was added; then the reaction mixture was incubated at room temperature for 6 h in an argon atmosphere. The unreacted biotin-PEG₆-azide was removed by gel filtration (1.5 × 20 cm Column of Sephadex G-50). The size of liposomes during the Staudinger ligation was monitored over time by using 90Plus particle analyzer.

6.2.5 Preparation of Biotin-Liposome via Direct Liposome Formation

DSPC (43.2 mg, 54.7 μ mol), cholesterol (10.6 mg, 27.4 μ mol), DSPE-PEG₂₀₀₀-Biotin (2.5 mg, 0.83 μ mol) (2: 1: 1 % molar ratio) in 3 mL of chloroform. The solvent was gently removed on an evaporator under reduced pressure to form a thin lipid film on the flask wall and kept in a vacuum chamber overnight. Then, the lipid film was swelled in the dark with 2.5 mL PBS buffer (pH 7.4), followed 10 freeze-thaw cycles of quenching in liquid N₂ and then immersed in a 50 °C water-bath to form multilamellar vesicle suspension. Finally, the crude lipid suspension was extruded through polycarbonate membranes (pore size 600, 200, and 100 nm, gradually) at a 60 °C to afford small unilamellar vesicles.

6.2.6 Immobilization of Biotin-Liposome onto Streptavidin Glass Slide

Immobilization of the biotinylated liposomes above was performed by incubating with streptavidin-glass slide (Xenopore Corp) in a 5 mg/mL of biotin-liposome suspension (total lipid concentration) at room temperature for 4 hrs, followed by removing the un-immobilized liposomes in the surface of glass slide. The glass slide was washed by rinsing with PBS buffer for 1 hrs and then replacing with new buffer solution, and repeated three times.

6.2.7 Liposome Array Based on Staudinger Ligation

The liposome prepared above was diluted to desired lipid concentration (2.0 mg/mL, total lipid concentration) by PBS (pH 7.4) buffer, then was printed onto azide-PEG₆-functionalized glass slide followed by incubating for 2.0 hrs at room temperature.

Next, the liposome-immobilized glass slide was washed by rinsing with PBS (pH 7.4) buffer for 2.0 hrs and repeated three times to remove unbound liposomes.

6.2.8 Staudinger Glyco-Functionalization of Immobilized Liposome

The liposome prepared above was printed onto azide-PEG₆-functionalized glass slide followed by incubating for 2.0 hrs at room temperature. Then the immobilized liposome carrying triphenylphosphine was incubated with 2-azideethyl-lactoside in PBS buffer (pH 7.4, 40 mg/mL) at room temperature for 2.0 hrs, followed by removing the glass slide from the reaction solution. The glass slide was then washed by rinsing with PBS (pH 7.4) buffer for 2.0 hrs and repeated three times.

6.2.9 Specific Lectin Binding onto Lactosylated Immobilized Liposome

The lactosylated liposome immobilized onto glass slide was incubated with lectin (Arachis hypogae, FITC-labeled, Sigma) in PBS (pH 7.4) buffer solution (50 g/mL) at room temperature for 2.0 hrs, followed by removing the glass slide from the reaction solution. The glass slide was then washed by rinsing with PBS (pH 7.4) buffer for 2.0 hrs and repeated three times.

6.2.10 OG488 Labeling of rTM₄₅₆

TM₄₅₆ was labeled with OG488 succinimidyl ester as described with modifications [33]. Briefly, to a solution of 150 μ L of carbonate-bicarbonate buffer (pH 9.0), 50 μ L of OG488 solution (10 mg/mL in DMSO) was added, followed by 150 μ L

aqueous TM₄₅₆ solution (2 mg/mL). The mixture was gently vortexed at room temperature in the dark for 2 hrs. After the coupling reaction, the unreacted OG488 was removed by dialysis using centrifuge devices with a cutoff molecular weight of 10,000 Dalton.

6.2.11 rTM Conjugation to Immobilized Liposome by Staudinger Ligation

The liposome prepared above was printed onto azide-PEG₆-functionalized glass slide followed by incubating for 2.0 hrs at room temperature. Then, the immobilized liposome carrying triphenylphosphine was incubated with rTM₄₅₆ labeled by OG488 in Tris-HCl buffer (pH 7.4, 0.5mg/mL) at room temperature for 2.0 hrs, followed by removing the glass slide from the reaction solution. The glass slide was then washed by rinsing with PBS (pH 7.4) buffer for 2.0 hrs and repeated three times. Fluorescence imaging of glass slide was performed to explore whether rTM₄₅₆ was conjugated to immobilized liposome by using a Typhoon 9410 Variable Mode Imager (Amersham Biosciences, USA).

6.2.12 Measurement of Releasing Kinetics of 5, 6-Carboxyfluorescein from Liposome

5, 6-carboxyfluorescein (CF) released from free liposomes, immobilized liposomes, and glycosylated immobilized liposomes in PBS (pH 7.4) buffer at room temperature was measured over time. The excitation and emission wavelengths of 5, 6-

CF were 497 and 520 nm, respectively. The variation of the fluorescent intensity with release time was calculated according to the equation below

$$\text{Fraction of CF remaining in liposomes} = 1 - F/F_0$$

where F is the fluorescent intensity measured at any time during the experiment and F_0 is the total fluorescent intensity measured after disrupting liposomes completely with 0.5% Triton X-100 in PBS (pH 7.4) buffer.

6.3 Results and Discussion

6.3.1 Chemically Selective Liposome Biotinylation and Its Immobilization

Streptavidin/biotin-based liposome immobilization has been widely used by synthesizing biotin-presenting liposome [34-36]. Conventionally, the biotin anchor group modified liposome is synthesized by direct liposome formation method, in which the biotin-lipid is mixed with all other lipid components to afford a liposome with biotin oriented both outside and enclosed aqueous compartment. In the present study, we explored azide reactive pre-prepared liposome carrying PEG-triphenylphosphine for chemically selective liposome surface biotinylation through Staudinger ligation with azide-containing biotin [Figure 6.3]. First, the terminal triphenylphosphine carrying anchor lipid DSPE-PEG₂₀₀₀-triphenylphosphine 1 was synthesized by amidation of commercially available DSPE-PEG₂₀₀₀-NH₂ with 3-diphenylphosphino-4-methoxycarbonylbenzoic acid NHS active ester synthesized as described in our previous study [15]. Next, small unilamellar vesicles composed of phospholipids (DSPC) and cholesterol (2:1 mol ratio) and 1.0 mol % of the anchor lipid 1 were prepared by rapid

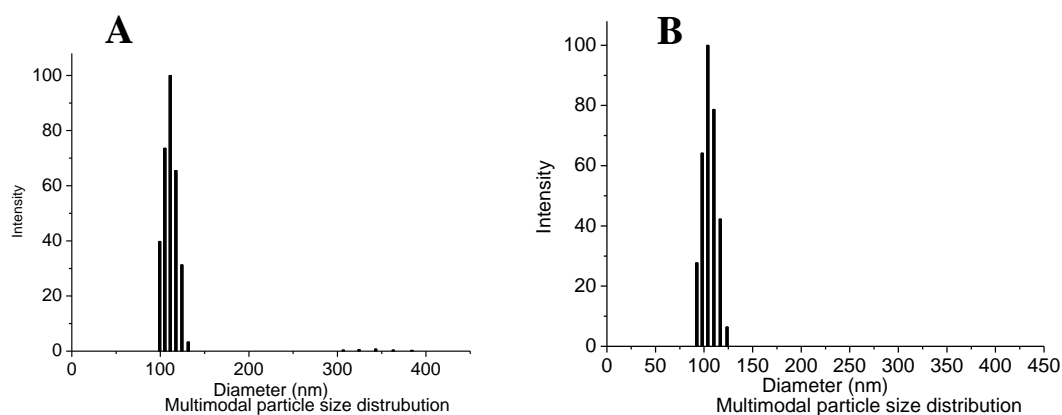


Figure 6.4 DLS Monitoring of Size Change of Liposome before Biotinylation (A) and after Biotinylation Reaction (B)

Next, streptavidin binding assay was examined to determine the success of the biotinylation and whether the grafted biotin residues are easily accessible at the surface of liposomes. It is well known that one streptavidin molecule is able to bind four biotin molecules and the presence of streptavidin could induce aggregation of surface biotinylated liposome. The biotin/streptavidin combination measurement was performed by incubating streptavidin with the biotinylated liposome in PBS buffer (pH 7.4) at room temperature. After 2 hrs, streptavidin-induced aggregation of the biotinylated liposomes was confirmed by DLS (Figure 6.4A), while there was no aggregation observed for the liposomes without biotinylation (Figure 6.4B). Furthermore, the presence of free biotin (5.0 mM) prevented aggregates formation (not shown), confirming that the aggregation was due to the specific recognition of the biotin residues on the surface of the liposome by streptavidin. These results indicated that the liposome surface has been biotinylated successfully and grafted biotin on the liposome surface was easily accessible. Similar

result was obtained with the direct-formed biotin-liposome as positive control (Figure 6.5C).

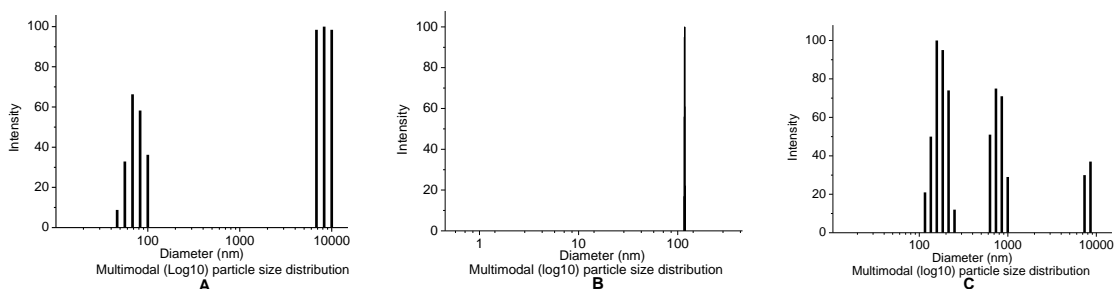


Figure 6.5 DLS Monitoring of Streptavidin Binding Assays of Biotinylated Liposomes: Post Biotinylated Liposomes (A), Plain Liposomes without Biotin (B) and Direct Biotinylated Liposomes (C)

Immobilization of the biotinylated liposome was performed by incubating with streptavidin-coated glass slide (Xenopore Corp) in PBS buffer (pH 7.4) at room temperature for 2 hrs followed by washing with PBS buffer (pH 7.4) three times. To confirm the immobilized liposome on the glass slide surface, fluorescent imaging study was examined with post biotinylated liposome and direct biotinylated liposome, respectively. To visualize liposomes in the fluorescence image, DSPE-NTB was doped in both liposomes as component for detecting by a microplate reader. As shown in Figure 4, both post biotinylated liposome (Figure 6.6A) and direct biotinylated liposome (Figure 6.6B) yielded a uniform fluorescence image, while there was no apparent fluorescence image observed for the nonbiotinylated liposomes (Figure 6.6C). Taken together, these results indicated that the immobilization of intact liposomes was achieved with biotin anchor.

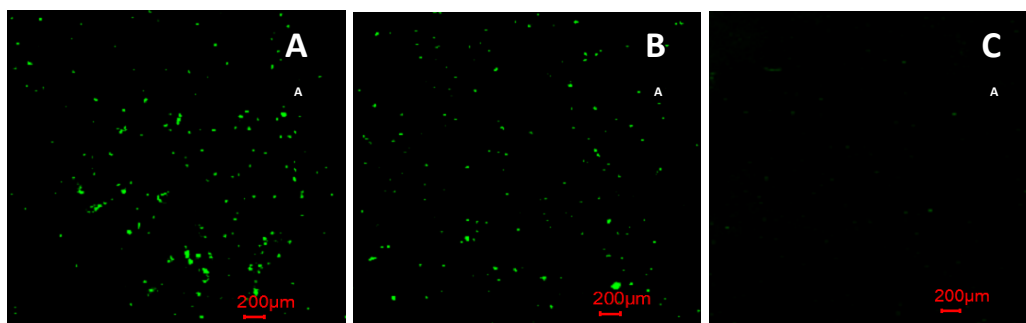


Figure 6.6 Fluorescence Image of Immobilized Liposomes onto Streptavidin-Coated Glass Slides: Post biotinylated liposomes (A), Direct Biotinylated Liposomes (B) and Plain Liposomes without Biotin (C) Doping with DSPE-NTB

To examine whether the immobilization reaction condition could provoke liposome disruption, fluorescent dye releasing kinetics from the liposome during immobilization reaction was investigated. Briefly, we have exposed our standard conditions to the same type of liposomes but with encapsulated selfquenching concentration (85 mM) of 5,6-CF. On the basis of the fluorescence quenching determinations (Figure 6.7A), we could demonstrate that less than 7.2 % leakage of the total loading was triggered during the immobilization reaction (2.0 hrs) compared with 5.2 % leakage for the same liposome during storage without reaction (2.0 hrs). The leakage percentiles were calculated by destroying liposomes with surfactant 0.5 % Triton X-100 in PBS (pH 7.4) buffer to release all 5,6-CF encapsulated after 2.0 h reaction. These results indicated that the immobilization reaction condition is harmless for liposome integrity. Continually, the subsequent fluorescent dye releasing kinetics over time for the non-destroyed immobilized liposomes was examined to verify the stability of the intact immobilized liposome in PBS (pH 7.4) buffer at room temperature. It was found that immobilized liposome showed a constant leakage of approximately 25 %/day

for three days (Figure 6.7B), whereas free liposome in PBS (pH 7.4) solution showed a leakage rate of approximately 20 %/day (Figure 6.7C), which is slightly less than that of immobilized liposome. The extended fluorescent dye releasing results further demonstrated that intact liposomes had been successfully immobilized onto the glass slide and showed sustained stability as well.

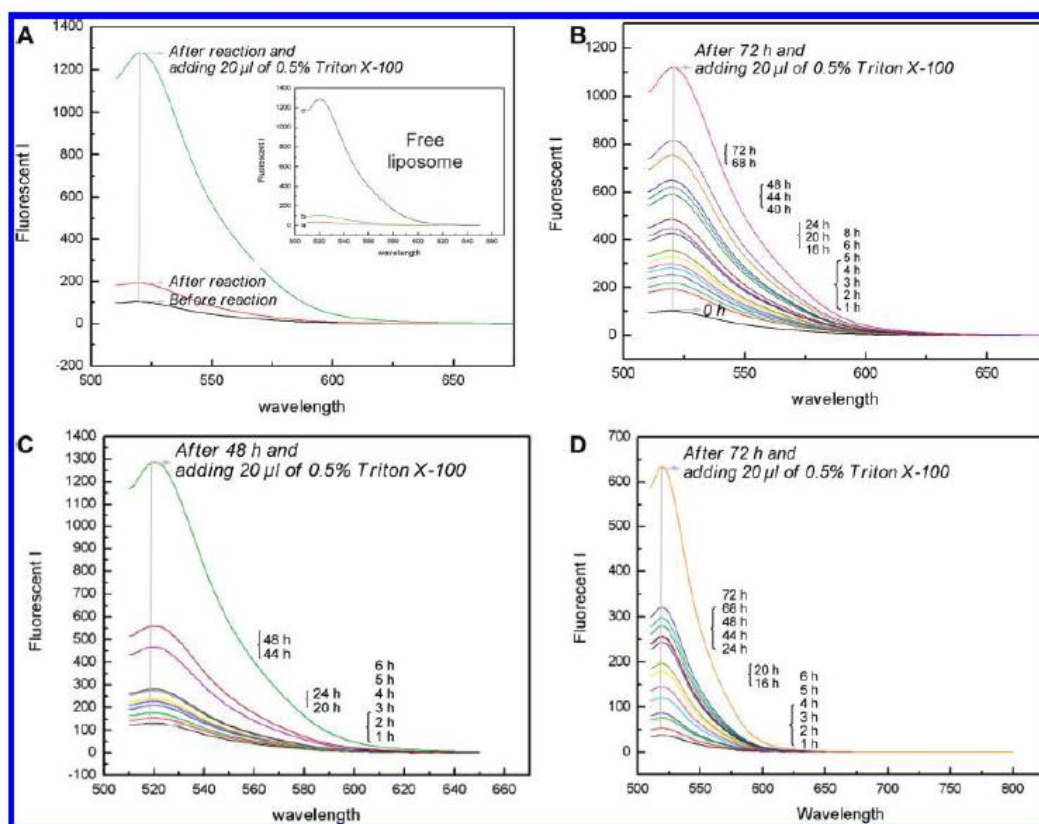


Figure 6.7 5,6-CF Releasing from Liposome during the Immobilization Reaction: before Reaction, after Reaction, and after Adding 20 μ L of 0.5 % Triton X-100 (A); the 5,6-CF Releasing Kinetics from Immobilized Liposome (B); the 5,6-CF Releasing Kinetics from Free Liposome (C); and the 5,6-CF Releasing Kinetics from Glycosylated Immobilized Liposome (D) during Storage in PBS (pH 7.4) Buffer at rt during 0-72 hrs, and after Adding 20 μ L of 0.5 % Triton X-100 at the Final Point.

To provide further evidence that immobilized liposomes remain intact on the glass slide surface, atomic force microscope (AFM) was used since it is a powerful tool for

investigating surface-related physical and chemical properties in nanoscale. As shown in Figure 6.8, typical AFM images of immobilized liposomes have been observed for post biotinylated liposome (Figure 6.8A) and direct biotinylated liposome (Figure 6.8B) as well, while there were no immobilized liposomes observed for the non-biotinylated liposomes (Figure 6.8C). These results further confirmed the successful immobilization of intact liposomes via biotin anchor.

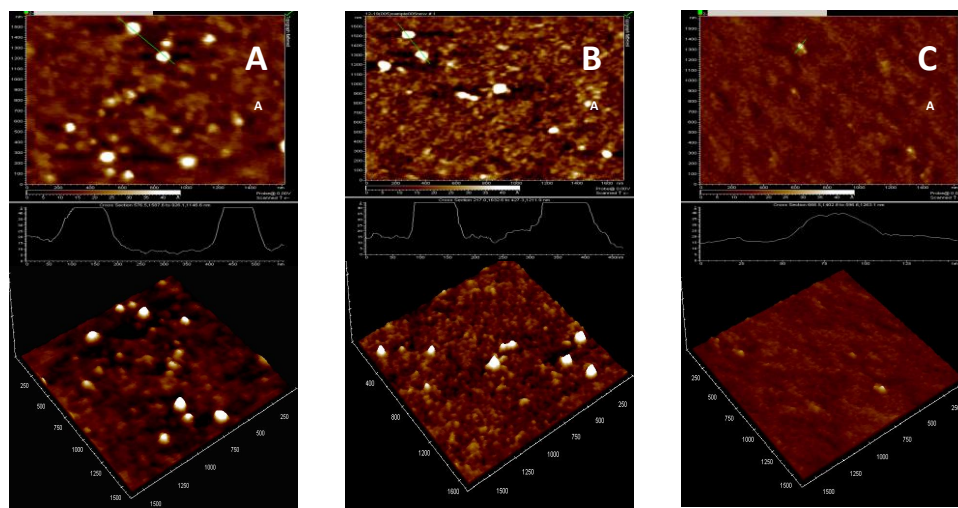


Figure 6.8 AFM Image Study of Immobilized Liposomes onto Streptavidin-Coated Glass Slides: Post Biotinylated Liposomes (A), Direct Biotinylated Liposomes (B) and Plain Liposomes without Biotin (C)

6.3.2 Chemically Selective Liposomal Microarray Fabrication

Liposome microarrays are versatile tools in biomedical research, as they can be used for applications in membrane biophysics, biotechnology, and colloid and interface science [7]. Most liposome microarrays were fabricated through bioaffinity between anchoring group on the liposome surface and the counterpart group on the solid surface, such as biotin/streptavidin⁷ and DNA hybridization [37]. In the current study, we

explored chemically selective liposome microarray and its rTM conjugation performed by the Staudinger ligation of a performed liposome carrying PEG-triphosphine with azide-functionalized solid surface. The azid-PEG₆-glass slide was used as model surface for liposome microarray and was prepared by amidation of commercially available amine glass slide (Xenopore, Co) with azido-PEG₆-COO-NHS (Figure 6.9)

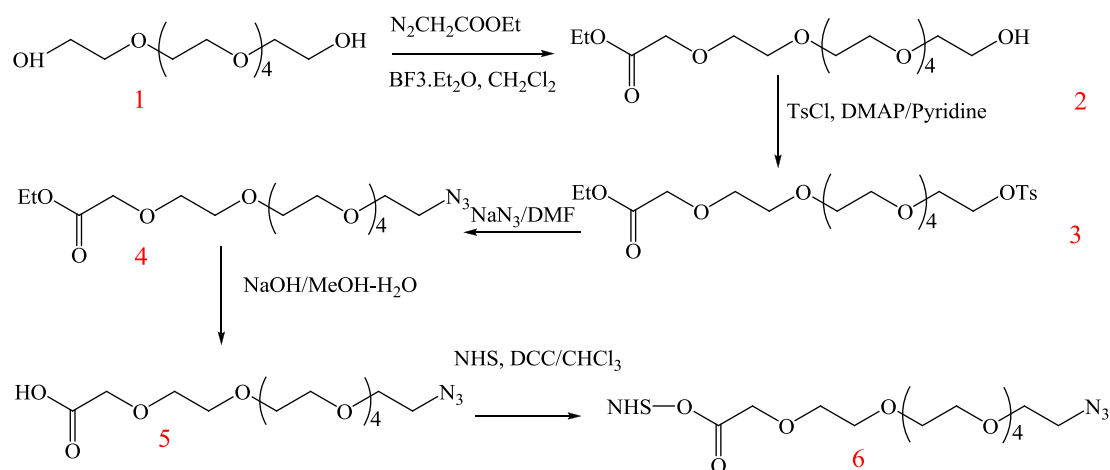


Figure 6.9 Scheme for Synthesis of Azido-PEG₆-COO-NHS

In order to confirm the intact liposome immobilized on the glass slide surface, fluorescence imaging study was conducted by doping DSPE-Rodamine (1 mol%, Avanti Polar Lipid, Inc) in the liposome lipid bilayer so as to label lipid membrane and by encapsulating 5,6-carboxyfluorescein (5,6-CF, Sigma) (50 mM) into the liposome so as to image the inner compartment of the liposome. As results, either detecting 5,6-CF (Figure 6.10A) or Rodamine (Figure 6.10B) yielded fluorescence image of the immobilized liposome for azide-PEG glass slide treated with liposomes carrying anchor group triphenylphosphine, while there was no apparent fluorescence image observed for azide-PEG glass slide treated with liposomes without anchor group triphenylphosphine (Figure 6.10C). These results indicated that the immobilization of intact liposomes was

achieved through Staudinger ligation. Next, glyco-modification was performed by incubation of the immobilized liposomes carrying triphenylphosphine left over on the liposome exterior surface with 2-azideethyl-lactoside [38] in PBS buffer (pH 7.4) at room temperature under an argon atmosphere for 2 hrs. Specific lectin binding assay was investigated to confirm the success of glycosylation and whether the grafted lactose residues are easily accessible at the surface of the immobilized liposomes. The binding assay was conducted by incubating lactosylated arrayed liposomes in the solution of - galactose binding lectin (*Arachis hypogae*, 120 kDa, FITC-Labeled, Sigma) in PBS (pH 7.4) buffer at room temperature for 2.0 hrs, followed by washing with PBS (pH 7.4) buffer three times. As shown in Figure 5, the specific binding of fluorescent labeled lectin was observed on the lactosylated immobilized liposome spots (Figure 6.10E), while there was no apparent fluorescence image observed for liposomes with anchor group triphenylphosphine unmodified (Figure 6.10D) and glycosylated liposome with lactose pre-incubated lectin (Figure 6.10F) . These results indicated specific binding of lectin onto the lactosylated arrayed liposomes.

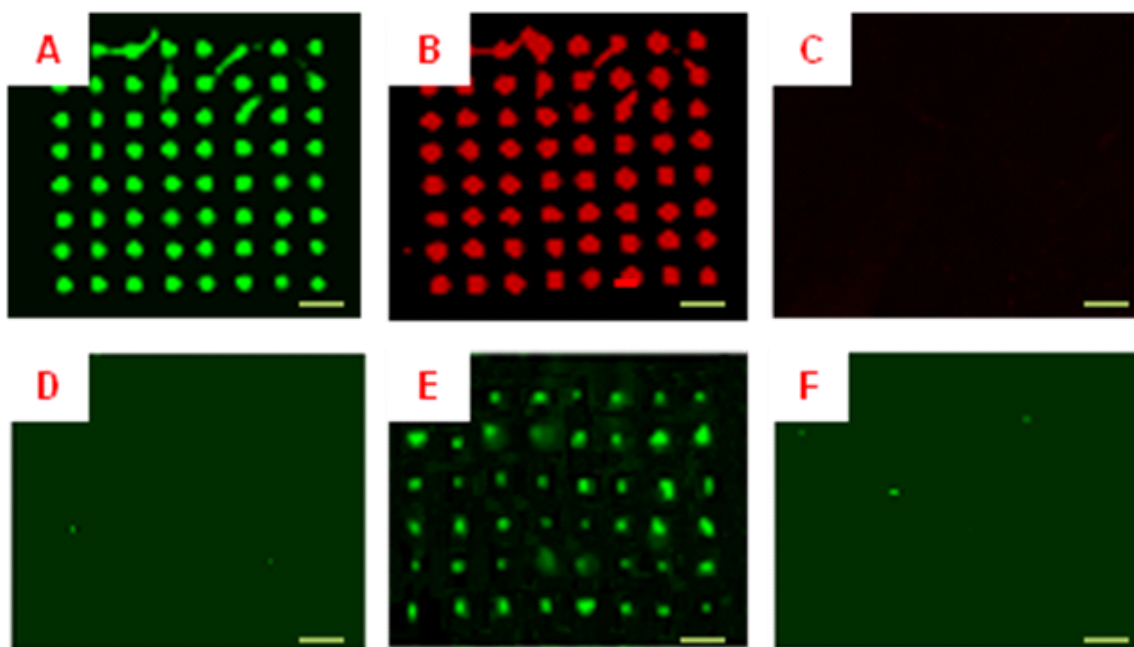


Figure 6.10 Fluorescence Images of Arrayed Liposomes: Selectively Exciting 5,6-CF Encapsulated in the Liposome (A) and for Selectively Exciting PE-Rhodamine Embedded in the Liposome Membrane (B), and Liposomes without Anchor Group Triphenylphosphine (C); Fluorescence Image of Lectin (FITC-labeled *Arachis hypogaea*) Binding onto the Arrayed Liposome: Liposomes with Anchor Group Triphenylphosphine (D), Glycosylated Liposome (D), and Glycosylated Liposome with Lactose Pre-incubated Lectin (E): Bar Size: 500 nm

6.3.3 rTM456 Conjugation to Immobilized Liposome by Staudinger Ligation

Next, liposome microarray and its rTM conjugation were designed as figure 6.11. In the step I, immobilization of the preformed liposomes was performed by incubating azide-PEG-glass slides with preformed liposomes in Tris-HCl buffer, followed by addition of MeO-PEG₂₀₀₀-triphenylphosphine to quench the azide group left on the glass slide. The unreacted liposomes were removed by washing with Tris-HCl buffer to afford immobilized liposome. In the step II, glass slide with immobilized liposome was incubated into OG488 labeled rTM₄₅₆ for 2hrs at room temperature under argon

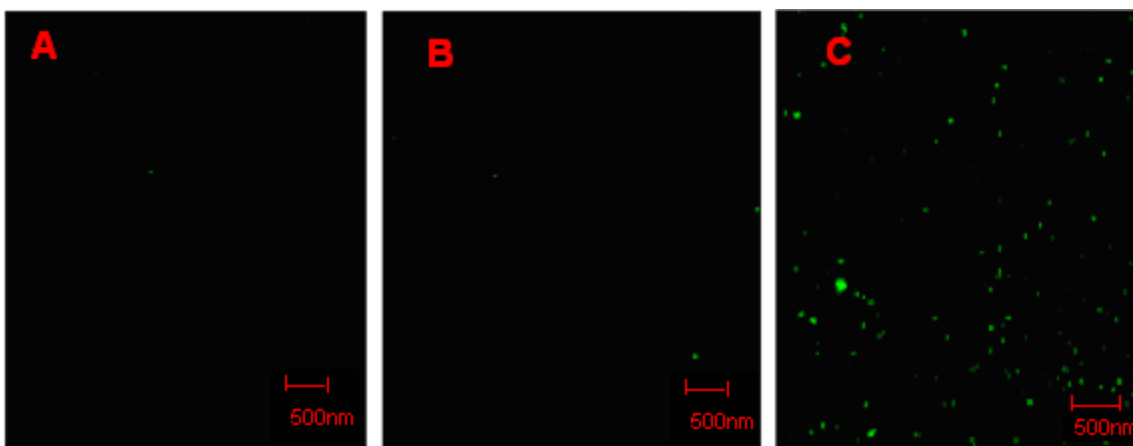


Figure 6.12 Fluorescent Image of rTM Conjugation to Immobilized Liposome: Positive control (Non-functionalized Glass Slide) (A), Negative Control (without Anchor Lipid) (B) and Immobilized Liposomal rTM Conjugate (C).

6.4 Conclusion

An azide reactive liposome has been developed for efficient and chemically selective liposome surface modification and glyco-liposome microarray fabrication applications. Specifically, microarray of liposome carrying triphenylphosphine onto azide-modified glass slide and further glyco-modification with azide-containing carbohydrate were demonstrated via Staudinger ligation. The high specificity and efficiency and biocompatible reaction condition natures of this liposome biotinylation approach make it an attractive alternative to all currently used protocols for liposome surface functionalization. Notably, since there is no catalyst used in modification and immobilization reaction, therefore, there is no concern of catalyst left in the resultant liposome that is common problem in other liposome modification methods. The reported method will provide a useful platform in the application of immobilized liposome system for a variety of biomedical researches and practices.

6.5 References

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CHAPTER VII

SUMMARY AND FUTURE PROSPECT

7.1 Summary of Current Work

Cardiovascular diseases are still most like reasons for death in United States although lots of attentions and efforts have been achieved to fight against them. Antithrombotic agents that prevent blood clotting have been used for both the prevention and treatment of active vascular thrombosis. However, current antithrombotic therapies have met limited success due to the risk of serious bleeding, hemorrhagic complication. In present study, our goal of the proposed research is to develop recombinant thrombomodulin (rTM)-based antithrombotic agents by synthesizing TM-liposome conjugate that mimics the native endothelial TM's antithrombotic mechanism. We hypothesize that combination of antithrombotic membrane protein TM into lipid membrane mimetic assembly (liposome) through recombinant and bioorthogonal conjugation techniques provides a rational strategy for generating novel and potential antithrombotic agent. The major accomplishments are briefly summarized below.

First, a chemo- and bio-orthogonal liposome surface functionalization through Staudinger ligation reaction has been developed. Briefly, an unprotected carbohydrate

derivative with an azide group was conjugated to the surface of vesicles presenting a synthetic lipid carrying a terminal triphosphine function. The liposomes were kept integrity during the reaction as measuring liposome size by dynamic light scattering (DLS) and monitoring fluorescent dye releasing kinetics. Moreover, the successful liposome surface functionalization was assessed by agglutination experiments using lectin, which show that the grafted galactose residues were perfectly accessible on the surface of liposome. This versatile approach, which is particularly suitable for the ligation of water-soluble molecules and without catalyst, is anticipated to be useful in the coupling of many other ligands onto liposome surface.

Second, to develop recombinant thrombomodulin (rTM)-based antithrombotic agents that mimics the native endothelial TM's antithrombotic mechanism, natural amino acid-azidohomoalanine was successfully incorporated into TM at the C terminal by directly acceptance of methionine analogs under help of Methionine-tRNA synthetases. This incorporation of azidohomoalanine provides an azide moiety for site specific conjugation through Staudinger ligation and Click chemistry, and makes an alternative to explore protein interaction and the effect of cell membrane to integral protein by membrane mimics.

Third, with azido-functionalized TM in hand, chemo-and bio-orthogonal methods including Staudinger and Click chemistry have been explored to synthesize liposomal rTM conjugate, respectively. In this study, protein C activation activities of the liposomal rTM conjugates were evaluated. His-TM₄₅₆-N₃ and TM₄₅₆-N₃ do not have apparent loss of activity while after conjugation, the value of k_{cat}/K_M of rTM-liposome conjugate has a twice to which of His-TM₄₅₆-N₃ or rTM₄₅₆-N₃ although there was no apparent change of

k_{cat} , this change of k_{cat}/K_M mainly resulted from decreasing of K_M which is responsible for affinity of rTM for protein C. This result was consistent with a previous report in which the enhancement of k_{cat}/K_M was from increase of affinity of rTM for protein C. This increasing of affinity may be the result of using liposome as a platform which has a beneficiary effect on the configuration of the conjugated rTM for enhancing thrombin and protein C binding. Therefore, the proposed membrane-mimetic re-expression of membrane protein domains onto liposome will provide a rational design strategy for facilitating studies of membrane protein functions and generating a membrane protein-based drug

In addition, the azide reactive liposome has been developed for efficient and chemically selective liposome surface modification and glyco-liposome microarray fabrication applications. Specifically, microarray of liposome carrying triphenylphosphine onto azide-modified glass slide and further glyco-modification with azide-containing carbohydrate were demonstrated via Staudinger ligation. The high specificity and efficiency and biocompatible reaction condition natures of this liposome biotinylation approach make it an attractive alternative to all currently used protocols for liposome surface functionalization. Notably, since there is no catalyst used in modification and immobilization reaction, therefore, there is no concern of catalyst left in the resultant liposome that is common problem in other liposome modification methods. The reported method will provide a useful platform in the application of immobilized liposome system for a variety of biomedical researches and practices.

7.2 Future Prospects

Although recombinant thrombomodulin (rTM)-based antithrombotic agents that mimic the native endothelial TM's antithrombotic mechanism have been successfully developed via chemo-and bio-orthogonal methods including Staudinger and Click chemistry, there still remain several prospects to explore before becoming an novel and potential antithrombotic agent. Further investigation will seek to define the capacity of the rTM-liposome to limit thrombus formation both in vitro and in vivo and to determine their pharmacokinetics. The following researches are expected to be conducted continually.

7.2.1 Evaluation of Anticoagulant Activity of rTM-Liposome Conjugates

Activated partial thromboplastin time (aPTT) and thrombin-clotting time (TT) are common method to analyze coagulation disorder and first-order method to assay anticoagulant therapy. Therefore, aPTT and TT should be performed to determine the anticoagulant property of liposomal rTM conjugates.

7.2.2 Antithrombotic Activity Assay of rTM-Liposome

Evaluation of antithrombotic activity of TM in vivo is another important way to demonstrate its efficacy and therapeutically promising application. A method for antithrombotic activity assay by tissue factor-induced microthromboembolism in mice should be investigated.

7.2.3 Measurement of the Circulating Half-life of rTM Liposome

To remain appropriate drug retention time, namely circulation lifetime in vivo, is very important to keep the maximum effect with minimizing the risk of toxicity. Lots of novel drug candidates suffered from relatively short half-time and lose the opportunity to develop a clinical drug. Particularly, to prolong life time is more challenging for protein based drugs compared to traditional small drug molecules due to living system clearance as well as enzymatic digestion. In the future, measurement of the circulating half-time of rTM-liposome should be performed to assay the effect of liposome and PEG on half-time of liposomal rTM conjugate. ELISA and functional APC assay can be performed to assay half-time of liposomal rTM conjugate according to previous study.